Human intestinal microbiota and metabolites they produce in relation to host health

A Thesis Presented to the National University of Ireland for the Degree of Doctor of Philosophy

By

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DECLARATION

I hereby certify that this material, which I submit for assessment on the programme of study leading to the award of Ph.D., is entirely my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

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ABSTRACT

The aim of this thesis was to identify selected potential probiotic characteristics of *Bifidobacterium longum* strains isolated from human sources, and to examine these characteristics in detail using genomic and phenotypic techniques. One strain in particular *Bifidobacterium longum* DPC 6315 was the main focus of the thesis and this strain was used in both the manufacture of yoghurt and an animal study. In total, 38 *B. longum* strains, obtained from infants and adults, were assessed *in vitro* for the selected probiotic traits using a combined phenotypic and molecular approach. Differentiation of the 38 strains using amplified ribosomal DNA restriction analysis (ARDRA) into subspecies indicated that of the 38 bifidobacterial strains tested, 34 were designated *B. longum* subsp. *longum* and four *B. longum* subsp. *infantis*.

The results of the screening analysis revealed that of the 38 *B. longum* strains tested, 15 *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain displayed an exopolysaccharide (EPS) phenotype in mMRS with 7% (w/v) lactose agar and broth, and 24 *B. longum* subsp. *longum* and two *B. longum* subsp. *infantis* strains displayed a positive PCR product using specific primers for a previously identified priming glycosyltransferase (pGTF) of a bifidobacterial EPS operon. Nine *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain converted free linoleic acid to *cis*-9, *trans*-11 (*c*9, *t*11) conjugated linoleic acid (CLA), and although lantibiotic production was not confirmed by the methods employed in this study, PCR analysis using primers specific to a previously identified lantibiotic structural gene *lanA* indicated the presence of the *lanA* gene in five *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain. *B. longum* DPC 6315 exhibited an EPS phenotype and converted free linoleic acid to *c*9, *t*11 CLA *in vitro* and whilst it did not produce a detectable bacteriocin by the methods employed in this study, it contained the EPS priming glycosyltranferase gene *wblE* and *lanA* gene.

The sequencing of the draft genome of *B. longum* DPC 6315 revealed it is 2.4 Mb in size with a 59% G + C content, encoding 10 scaffolds, comprised of 90 large contigs. Four predicted rRNA operons were found to be encoded within the genome. In addition, two plasmids of approximately 10.2 kb and 8.2 kb respectively were identified which were found to be almost identical (~99.5%) to previously isolated *B. longum* plasmids pNAC3 and pNAL8L. These plasmids were found to contain genes encoding replication, mobilization and transposase functions along with a host of hypothetical proteins. The draft genome of *B. longum* DPC 6315 also contains the genes necessary for EPS, lantibiotic production and myosin cross-reactive antigen (*mcra*) genes believed to be involved in CLA production in *Lactobacillus reuteri* PYR8. *B. longum* DPC 6315 was found to produce an EPS consisting of 12:6:3:1 of mannose: glucose: galactose: rhamnose and to display a ropy phenotype in mMRS with 7% (w/v) lactose, sucrose and glucose broth. A putative EPS operon (~16 kb) was identified in the genome of *B. longum* DPC 6315 – this potentially contained all the genes necessary for EPS biosynthesis i.e. chain length determination and export. These included genes coding for glycosyltransferases including a pGTF gene (*wblE*) and genes *wblB and wblC* involved in the synthesis of the EPS repeating subunit, and the genes *wblA* involved in EPS repeat unit polymerization and *wblD* involved in chain length determination.

The influence of the EPS-producing adjunct strain, *Bifidobacterium longum* DPC 6315 on the functional properties of yoghurt was examined. *In situ* production of EPS by *B. longum* DPC 6315 during product manufacture and up to 14 days of cold storage at 4°C was found to significantly ($P<0.05$) increase (from 36.9 (± 2.9) mg/l on day 0 to an average of 51.4 (± 9.4) mg/l EPS on day 7). In contrast, the concentration of EPS in the EPS containing yoghurt slightly declined on day 14, 21 ($P<0.01$) and 28

(P<0.01) of cold storage, to an average of 19.3 (± 4.4) mg/l EPS on day 28. The performance and viability of the yoghurt cultures were not affected by addition of *B. longum* DPC 6315, with *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* detected at 1.7×10^7 CFU/g and 2.8×10^5 CFU/g, respectively, following 28 days of storage at 4°C. The viability of the EPS-producing *B. longum* DPC 6315 was 4.7×10^6 CFU/g after 28 days of storage at 4°C, which is above the minimum of 10^6 CFU/g recommended by the FAO/WHO guidelines (FAO/WHO, 2002) for a probiotic bacterium in a fermented food. During yoghurt manufacture and at 14 days of storage at 4°C, a significant decrease (P<0.05) in syneresis was obtained in yoghurt containing the EPS producing strain compared with the control. No significant difference was found in viscosity after fermentation and during 28 days of storage at 4°C between the yoghurt manufactured with the EPS-producing *B. longum* DPC 6315 and the control yoghurt. Confocal laser scanning microscopy (CLSM) of unstirred yoghurt samples demonstrated that the EPS was present around the edges of the pores of the yoghurt network but not in the control yoghurt. This study demonstrates that the use of *B. longum* DPC 6315 as an adjunct culture during yoghurt manufacture resulted in lower levels of syneresis and higher levels of EPS, and as such use of the adjunct offers some technological improvements to the product over and above its probiotic characteristics.

Finally, *B. longum* DPC 6315 was fed along with a high fat diet enriched with α-linolenic acid (test group) to BALB/c mice. In the control or unsupplemented group, BALB/c mice were fed with the high fat diet enriched with α-linolenic acid but without the strain. *B. longum* DPC 6315 was found to survive passage through the mouse GIT. Following 6 weeks of feeding *B. longum* DPC 6315, it was recovered at ~4.4×10^4 CFU/g faeces in the test group. Fatty acid composition analysis revealed

that mice that received *B. longum* DPC 6315 had significantly lower ($P<0.01$) levels of linoleic acid and γ-linolenic acid ($P<0.001$) acid in the liver, and stearic acid ($P<0.05$) levels in the brain compared with unsupplemented mice. In contrast, mice that received *B. longum* DPC 6315 had significantly higher concentrations of stearic acid ($P<0.05$), EPA, ($P<0.05$) and DPA, ($P<0.05$) in the liver. The microbial diversity of the caecal samples from the two mouse groups was determined using 16S rRNA amplicon pyrosequencing technology. In total 425,285 sequences were obtained from 19 caecal samples collected from the two mouse groups. The mouse microbiota samples taken from both groups fed *B. longum* DPC 6315 and α-linolenic acid and the unsupplemented group were dominated by *Firmicutes* (72% and 61% respectively), followed by *Bacteriodetes* (17% and 29% respectively). *Firmicutes* were found at a significantly ($P=0.0076$) higher (0.2 fold increase) relative abundance in the group that received *B. longum* DPC 6315, while *Bacteriodetes* were found at a significantly ($P=0.0057$) higher (0.7 fold increase) proportions in the unsupplemented group. At the genus level, *Alistipes* was detected at a significantly ($P=0.04$) higher (0.8 fold increase) relative abundance in the unsupplemented group, while *Roseburia* was detected at a significantly ($P=0.04$) higher (0.5 fold increase) relative abundance in the group that received *B. longum* DPC 6315. In conclusion, supplementation with *B. longum* DPC 6315 led to significant differences in fatty acid composition in both the murine liver and brain tissues compared to supplementation with α-linolenic acid alone. *B. longum* DPC 6315 supplementation also led to significant differences in caecal microbiota composition at both the phylum and genus level.

Overall, the results presented in this thesis outlined some of the potentially probiotic traits present in *B. longum* strains, the presence of these genes in the genome of *B. longum* DPC 6315, and the capacity of *B. longum* DPC 6315 to influence the

functional properties of yoghurt and the fatty acid composition and overall caecal microbiota in a murine model *in vivo*.

ABBREVIATIONS

AA	Amino Acid
ABC	ATP-Binding Cassette
ACT	Artemis Comparison Tool
ATP	Adenosine Triphosphate
ARDRA	Amplified Ribosomal DNA Restriction Analysis
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	base pairs
C	Cytosine
CFU	Colony Forming Unit
CLA	Conjugated Linoleic Acid
CLNA	Conjugated Linolenic Acid
CLSM	Confocal Laser Scanning Microscopy
Da	Dalton
DHA	Docosahexanoic Acid
dH_2O	Distilled Water
DM	Maltodextrin
DNA	Deoxyribonucleic Acid
DP	Degree of Polymerisation
DPA	Docosapentaenoic Acid
DPC	Dairy Products Research Centre
dTDP	Thymidine diphosphate
E_h	Oxidation-Reduction Potential
EPA	Eicosapentaenoic Acid

EPS	Exopolysaccharide
FAMEs	Fatty Acid Methyl Esters
FAO/WHO	Food and Agriculture Organisation of the United Nations and the World Health Organisation
Fig.	Figure
FOS	Fructo-Oligosaccharide
FOSHU	Food for Specified Health Uses
g	Gram
g	Gravitational Force
G	Guanine
GC-MS	Gas Chromatography-Mass Spectrometry
GIT	Gastrointestinal Tract
GLP	Glucagon-Like Peptide-1
GOS	Galactooligosaccharides
h	Hour
H^+	Hydrogen Ion
HCL	Hydrogen Chloride
HDL	High-Density Lipoprotein
HPLC	High-Performance Liquid Chromatography
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IS	Insertion Sequence
LA	Linoleic Acid
LAB	Lactic Acid Bacteria

LDL	Lower Density Lipoprotein
LPS	Lipopolysaccharide
MCRA	Myosin Cross-Reactive Antigen
MEGAN	MEtaGenome Analyser
Min	Minutes
mg/l	milligrams per litre
ml	milli-litre
mPa.S	milli Pascal Seconds
MRD	Maximum Recovery Diluent
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
MRS	de Mann, Rogosa, Sharpe
mMRS	modified MRS
Na^+	Sodium Ion
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NMR	Nuclear Magnetic Resonance
OFS	Oligofructose
ORF	Open Reading Frame
OTU's	Operational Taxonomical Units
PAANS	Pre-Acetylated Aldonitrile Acetates
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
PEP	Phosphoenolpyruvate
PFGE	Pulse Field Gel Electrophoresis

pGTF	priming Glycosyltransferase
ppm	parts per million
rpm	Revolutions per minute
rRNA	ribosomal RNA
RSM	Reconstituted Medium-Heat Skimmed Milk
s^{-1}	per second
SCFA	Short Chain Fatty Acids
SEM	Standard Error Means
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic Acid
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factors
TOBA	Taxonomic Outline of Bacteria and Archaea
tRNA	transfer RNA
UDP	Uridine Diphosphate
UPGMA	Unweighted Pair Group Method with Arithmetic Averages
UTP	Uridine-5'-Triphosphate
UV	Ultraviolet
WT	Wild-Type
w/v	Weight per volume
v/v	Volume per volume
YE	Yeast Extract
β-gal	Beta-galactosidase
µg	Micro-gram

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CHAPTER 1

Metabolic activities and probiotic potential of bifidobacteria.

This chapter has been published as a review article;

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1.1 ABSTRACT

It has been shown that the gut microbiota regulates fat storage in the body and that disturbances in its composition can lead to the development of certain metabolic disease states. Bifidobacteria are found among the resident microbiota in the gastrointestinal tract (GIT) and their metabolic activities have been shown to beneficially influence the human host. It has been reported that they inhibit intestinal colonisation by pathogenic microorganisms and have anti-carcinogenic, immunostimulatory, and anti-diarrhoeal properties, as well as aiding in the alleviation of lactose intolerance and ability to lower serum cholesterol levels in humans. One particular health promoting property of bifidobacteria is bioactive fatty acid production, which when ingested, may confer health benefits on the host. The major bioactive fatty acid produced by bifidobacteria is conjugated linoleic acid (CLA), of which *cis*-9, *trans*-11 (*c*9, *t*11) and *trans*-10, *cis*-12 (*t*10, *c*12) CLA are the main biologically active CLA isomers. The production of CLA by *Bifidobacterium* can also have a positive effect on the immune system of the human host leading to numerous health benefits. This is an example, where the metabolic activities of an ingested bacterium are beneficial to the host, rather than the direct interaction of the bacterium with the host.

1.2 INTRODUCTION

Since bifidobacteria were first isolated from the faeces of breastfed infants by Henry Tissier from the Pasteur Institute in 1899 (Scardovi & Trovatelli, 1969; Tissier, 1900; Yaeshima *et al*., 1992), they have come to be regarded as one of the most important bacterial groups associated with human health. Bifidobacteria are commonly used as probiotics for human consumption, as they are normal inhabitants of the GIT of humans (Simon & Gorbach, 1984). They have a long history of safe use in fermented milks (Ishibashi & Yamazaki, 2001) and are added to the human diet as food supplements. Bifidobacteria exert numerous positive effects on human health such as prevention of infection by pathogenic bacteria (Wang *et al*., 2004), immunostimulatory (Furrie *et al*., 2005) and anti-carcinogenic capabilities (Le Leu *et al*., 2010), protection against infectious diarrhoea, (Saavedra *et al*., 1994), lowering of serum cholesterol and alleviation of lactose maldigestion, all of which will be discussed below.

The expansion in bifidobacterial genomics has helped develop our understanding of their interactions within the human GIT and will lead to more interesting aspects of bifidobacterial physiology and their roles in the normal GIT being discovered in the future. This review will focus on a number of aspects of *Bifidobacterium* such as their classification, where they are found in nature, bifidobacteria's composition among the normal gut microbiota, their metabolic activities and how these activities impact on the human host. Also discussed, will be the role bifidobacteria can play in the regulation of host fat tissue composition, through the production of bioactive fatty acids. In addition, the use of bifidobacteria as a probiotic, and the future implications of bifidobacteria as a probiotic will be addressed in this review.

1.3 CLASSIFICATION

Bifidobacteria are Gram-positive polymorphic rods that can occur singly, in chains or in clumps. These bacteria are non-spore-forming, non-motile, and non-filamentous (Felis & Dellaglio, 2007). Their morphology ranges from uniform to branched bifurcated Y and V forms which are spatula or club shaped (Leahy *et al*., 2005). The branching nature of bifidobacteria is strain- and media-dependent (Tannock, 1999). They are obligate anaerobes (some strains can tolerate oxygen in the presence of carbon dioxide), (Simpson *et al*., 2004) and chemoorganotrophic (organisms that use organic compounds as their energy source). *Bifidobacterium* are saccharolytic and produce acid but not gas from a variety of carbohydrates.

Bifidobacteria were first found in faeces of breastfed infants (Tissier, 1900) and have also been isolated from breast milk (mainly *Bifidobacterium longum* followed by *Bifidobacterium animalis, Bifidobacterium bifidum*, and *Bifidobacterium catenlatum*), (Fanaro *et al*., 2003; Gueimonde *et al*., 2007). The original name, *Bacillus bifidus communis* (Tissier, 1900) signifies the branching morphology of the bacteria; bifidus in Latin meaning cleft in two parts (Ishibashi, 1997). Bifidobacteria are often difficult to isolate and grow in the laboratory, as they have special nutritional requirements (Arunachalam, 1999). Orla-Jensen (Jensen, 1924) first proposed that *Bacillus bifidus* be classified as a separate species under the genus name *Bifidobacterium*. The explanation for this was that species of bifidobacteria should constitute a separate genus, which would possibly form a link between lactic acid bacteria (LAB) and the propionic acid bacteria (Jensen, 1924). However, for much of the 20th Century there was still no taxonomic consensus for this new genus, and they were classified as members of the genera

Bacillus, Bacteroides, Nocardia, Lactobacillus and *Corynebacterium* among others, before the 8th edition of Bergey's Manual reclassified them as a general taxon and designated the genus *Bifidobacterium,* consisting of 11 species (Bergey *et al*., 1974).

Bifidobacteria are phylogenetically distinct from LAB with a G + C content ranging from 42% to 67% (Biavati and Mattarelli, 2001), but are often included in this group because of their metabolic capacities. Bifidobacteria belong to the phylum *Actinobacteria*, whereas other LAB such as *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus,* or *Weisella* are *Firmicutes* (Ishibashi, 1997). In the phylum *Actinobacteria*, the order *Bifidobacteriales* consists of two families, *Bifidobacteriaceae* and *Incertae* (Garrity *et al*., 2007). In the most recent *Taxonomic Outline of Bacteria* and *Archaea*, release 7.7 (TOBA 7.7), it is suggested that the *Bifidobacteriaceae* family be divided into five genera: *Bifidobacterium, Aeriscardovia, Gardnerella, Parascardovia,* and *Scardovia* (Garrity *et al*., 2007). The inclusion of the genera *Aeriscardovia, Parascardovia,* and *Scardovia* was suggested because of the different G + C contents and partial heat shock protein 60 gene sequences of three *Bifidobacterium* species, which are, *Bifidobacterium aerophilum, Bifidobacterium denticolens,* and *Bifidobacterium inopinatum*, respectively (Jian & Dong, 2002; Simpson *et al*., 2004). These three species are therefore not presently included in the *Bifidobacterium* genus. Bifidobacteria catabolise hexoses through a peculiar metabolic pathway involving the key enzyme fructose-6-phosphoketolase (EC 4.1.2.2), the fructose-6-phosphate pathway or the so-called *bifid shunt*. Before the reclassification of *Bifidobacterium* species into a new genus, this enzyme was considered a taxonomic character for the identification at the genus level

(Biavati & Mattarelli, 2001), but now it can be considered a taxonomic marker for the family *Bifidobacteriaceae.*

In the genus *Bifidobacterium,* several species are associated in groups that have been named according to the oldest name included in the group; these groups are ***Bifidobacterium adolescentis*** **group** (which includes *B. adolescentis, Bifidobacterium angulatum, B. catenulatum, Bifidobacterium dentium, Bifidobacterium merycicum, Bifidobacterium pseudocatenulatum,* and *Bifidobacterium ruminantium),* ***Bifidobacterium pullorum*** **group** (*Bifidobacterium gallinarum, B. pullorum,* and *Bifidobacterium saeculare),* ***Bifidobacterium asteroides*** **group** (*B. asteroides, Bifidobacterium coryneforme, B. indicum),* ***Bifidobacterium boum*** **group** (*B. boum, Bifidobacterium thermacidophilum, Bifidobacterium thermophilum*), ***Bifidobacterium pseudolongum*** **group** (*Bifidobacterium animalis, Bifidobacterium choerinum, Bifidobacterium cuniculi, Bifidobacterium gallicum, B. pseudolongum*). The species *Bifidobacterium breve* and *B. longum* form a couple, as well as *Bifidobacterium minimum* and *Bifidobacterium psychroaerophilum* (less supported). *B. bifidum, Bifidobacterium magnum, Bifidobacterium scardovii* and *Bifidobacterium subtile* form distinct branches (Felis & Dellaglio, 2007).

Probiotic properties which appear to be strain specific, have been found in some bifidobacterial strains that are not phylogenetically related, these strains belong to the species *B. adolescentis, B. animalis, B. bifidum, B. breve,* and *B. longum*. In the genus *Bifidobacterium* there are some taxonomic issues regarding the species *B. longum* and *Bifidobacterium infantis,* this has led to their union under *B. longum* and the reclassification in 2008 (Mattarelli *et al*., 2008) of three biotypes by molecular methods,

the *infantis* type, the *longum* type, and the *suis* type (Sakata *et al*., 2002). Also the interaction of *B. infantis* and *B. longum* with dentritic cells has led to some interesting results. The study performed by Young et al. (2004) found that ATCC/DSMZ cultures of these two species have different interactions with dendritic cells and should be kept distinct. The relationship between *B. animalis* and *B. lactis* is also debatable, with the latter being reclassified as *B. animalis* subsp. *lactis* (Masco *et al*., 2004). The differentiation between *B. indicum* and *B. asteroides* could be clearly established, but not so with *B. indicum* and *B. coryneforme* (Felis & Dellaglio, 2007).

1.3.1 Where *Bifidobacterium* species are found in the GIT

In general *Bifidobacterium* species inhabit the GIT of humans and animals where they coexist with a large variety of obligate anaerobes (Vaughan *et al*., 2000). The GIT is composed of different sections or subdivisions, beginning with the oral cavity, the stomach, the small intestine (duodenum, jejunum and ileum) and the large intestine (caecum, colon and rectum). A distinct bacterial community can be found in each section and bifidobacteria can be found all along the GIT (Crociani *et al*., 1996; Marteau *et al*., 2001; Nielsen *et al*., 2003; Rasic & Kurmann, 1983). Ten species have been isolated from human sources, *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. dentium, B. gallicum, B. longum* subsp. *longum* and *infantis, B. pseudocatenulatum* and *B. scardovii* (Biavati *et al*., 1984; Biavati *et al*., 1986; Gavini *et al*., 2001; Hoyles *et al*., 2002; Mangin *et al*., 1999; Matsuki *et al*., 1999; McCartney *et al*., 1996; Requena *et al*., 2002; Sakata *et al*., 2002). In total, species of bifidobacteria inhabit seven different ecological niches: the human intestine (Reuter, 1963), the human vagina (Verhelst *et al*.,

2005), the oral cavity (Okamoto *et al*., 2008; Scardovi & Crociani, 1974), food (Delcenserie *et al*., 2007), the animal GIT (Scardovi *et al*., 1979), sewage (Biavati *et al*., 1982), and the intestine of honeybees (Scardovi & Trovatelli, 1969), as shown in (Table 1.1). Two *Bifidobacterium* species, *B. asteroides* and *B. coryneforme* have been detected in the intestine of the honeybee, whereas *B. indicum* has been detected in the intestine of two other honeybee species, *Apis cerana* and *Apis dorsata* from the Philippines and Malaysia (Goulson, 2003; Killer *et al*., 2009b). Killer and colleagues (Killer *et al*., 2009b) were the first to isolate bifidobacteria from the digestive tract of bumblebees. In this study, it was predicted that bifidobacteria form an essential part of the bumblebee gut microbiota, and that because all of the species isolated in this study were different from the three species of the genus *Bifidobacterium* (*asteroides, coryneforme* and *indicum*) found in the intestine of honeybees, that they might belong to new species within the family *Bifidobacteriaceae.* In total, four bifidobacterial groups were isolated from the bumblebee intestine. The first of these groups to be proposed as a new species of *Bifidobacterium* was *Bifidobacterium bombi* sp. nov. (Killer *et al*., 2009a). Two new species of *Bifidobacterium* were also proposed, which were *Bifidobacterium actinocoloniiforme* sp. nov., and *Bifidobacterium bohemicum* sp. nov. (Killer *et al*., 2010a). Killer et al. (2010b) also proposed another new species of *Bifidobacterium* isolated from the bumblebee intestine which was *Bombiscardovia coagulans* gen. nov., sp. nov. Watanabe and colleagues, (2009) isolated a new species of *Bifidobacterium* from airag, which is a traditional fermented mare's milk product from Mongolia. This species was named *Bifidobacterium mongoliense* (Watanabe *et al*., 2009). Another new species of *Bifidobacterium* was isolated by Kim et al. (2010) from human faeces, from a healthy

27 year Korean male. This species was called *Bifidobacterium stercoris* sp. nov. (Kim *et al*., 2010).

The *Bifidobacterium* species which inhabit the human intestinal tract are distinct from those that inhabit animal intestines (Mitsuoka, 1984). Species that are representative of human origin include *B. longum*, *B. breve*, *B. bifidum*, *B. adolescentis* and *B. pseudocatenulatum*. Animal derived species include *B. pseudolongum*, *B. thermophilus* and *B. animalis.* Some animal species are host specific such as *B. magnum* and *B. cuniculi* which have only been found in rabbit faecal samples, *B. pullorum* and *B. gallinarum* in the chicken intestine and *B. suis* only in piglet faeces (Matteuzzi *et al*., 1971). *B. minimum* and *B. subtile* have only been identified in sewage.

1.4 BIFIDOBACTERIAL GENOMICS

The public genomic databases at present contain 18 complete bifidobacterial genomes, in addition to a large number of ongoing sequence projects (**http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi**). The first bifidobacterial genome to be sequenced and made public was *Bifidobacterium longum* NCC2705 (Schell *et al*., 2002). The genome was 2.26 Mb comparable to LAB genomes ranging from 1.8 to 2.6 Mb (Siezen *et al*., 2004). The sequencing of the *B. longum* NCC2705 genome was a considerable step forward, as previously there were less than 50 bifidobacterial protein sequences deposited in GenBank. The complete sequences of other *B. longum* genomes (*Bifidobacterium longum* DJO10A, (Lee *et al*., 2008), *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (Sela *et al*., 2008) and *Bifidobacterium longum* subsp. *longum* JDM301 (Wei *et al*., 2005) have become publicly available for genetic comparison with the NCC2705 genome. This has led to an understanding of the evolution within *Bifidobacterium* species, and has helped determine genes that form the bifidobacterial backbone or in other words are conserved in all strains. Variable regions of DNA sequence that determine strain specificity, and enable adaptation to a particular niche or lifestyle can be identified using comparative analysis on these recently published genomes.

The genome of *B. longum* NCC2705 (Schell *et al*., 2002) (GenBank accession number AE014295) is a 60% GC circular chromosome, which contains 4 ribosomal RNA operons, 57 transfer RNAs, 16 intact insertion sequence (IS) elements, integrated plasmid sequences (Schell *et al*., 2002), and a prophage-like element (Ventura *et al*., 2005). There are 1,727 proteins encoded within the genome. *B. longum* is a strict anaerobe it has no

aerobic respiratory components (Schell *et al*., 2002). It is a bacterium that has successfully adapted to living in the colon, and physiological traits relating to this can be seen in its genome (Schell *et al*., 2002). A large amount of the coding capacity of the genomes of *B. longum* NCC 2705 and *Bifidobacterium breve* UCC2003 (>8% of the genome) seems to be committed to the transport (mainly ABC-type transporters) and catabolism (glycosyl hydrolases) of mono-, oligo-, and poly- saccharides (Coutinho & Henrissat, 1999), traits that are present in other colonic inhabitants, such as *Enterococcus faecium* and *Bacteroides fragilis* (Flint, 2008). Genome evaluation has revealed some interesting bifidobacterial interactions with their hosts such as, many polypeptides which were discovered with homology to proteins involved in the production of glycoprotein-binding fimbriae, which may be valuable for adhesion and persistence in the GIT (Schell *et al*., 2002).

Many enzymes involved in carbohydrate metabolism were identified from the genome of *B. breve* UCC2003, which are similar to *B. longum* NCC 2705, including up to 40 glycosyl hydrolases whose possible substrates are a broad selection of oligo- and polysaccharides (Mayo *et al*., 2008). Many of these identified glycosyl hydrolases are believed to be active outside the cytoplasm; one of which is amylopullulanase, which gives UCC2003 the capacity to grow on starch and other related polymers (Ryan *et al*., 2005). Genome analysis has revealed that the biosynthetic capabilities of *Bifidobacterium* species have adapted to an environment where there is not a reliable extraneous source of amino acids, nucleotides and certain vitamins.

Bifidobacteria display minimal phylogenetic diversity and are characterised by a high degree of conservation and synteny across the entire genomes (Ventura *et al*., 2007).

The genome comparison of species with distinct eco-phenotypes such as the oral *B. dentium* and the intestinal *B. adolescentis* has identified breakpoint regions of genomic plasticity (Ventura *et al*., 2009a). A relative expansive autotrophy for amino acids, nucleotides, vitamins and cofactors and an extended capacity for degradation and utilisation of complex carbohydrates have been revealed from bifidobacterial genomics (Ventura *et al*., 2007). The carbohydrate sequestering capacity of bifidobacteria in the intestine is likely to establish a large competitive advantage over other bacterial species present.

Many mostly intracellular glycosylhydrolases involved in the initial degradation of complex carbohydrates such as arabinogalactans, arabinoxylans, starch, and similar polysaccharides are present in the bifidobacterial genome (Kim *et al*., 2008; Ryan *et al*., 2005; Scheffers & Pinho, 2005; Sela *et al*., 2008). Transport systems for the internalisation of structurally diverse carbohydrates are combined with these glycosylhydrolases, these were identified and found to contain docking sites for carbohydrate binding to the bacterial cell wall, which possibly prevents loss to nearby competitors (van den Broek *et al*., 2008). Different *Bifidobacterium* species have distinct capacities for the utilisation of complex carbohydrates; which would indicate an ecological specificity. This has been revealed by the discovery of an operon independently present in the genomes of *B. breve* strains commited to the breakdown of starch, amylopectin, and pullulan (Maze *et al*., 2007), which might clarify the corresponding abundance of *B. breve* in the infant microbiota (Ventura *et al*., 2004). With the determination of the complete genome of the oral *B. dentium* (2.7Mb), an interesting capacity to metabolise a wide range of simple sugars as well as complex

carbohydrates has been revealed, which would be related to the nutrient availability in the oral habitat (Ventura *et al*., 2009b).

1.5 GUT MICROBIOTA – COMPOSITION

The resident microbiota in the GIT is a heterogeneous microbial ecosystem harbouring up to 1×10^{14} colony-forming units (CFU) of bacteria (Berg, 1996; Morelli *et al*., 1998; Vaughan *et al*., 2000; Zboril, 2002) i.e. 10- to 100-fold more genes than the human genome and providing regulatory signals for immune and gastrointestinal maturation (O'Hara & Shanahan, 2006; Round & Mazmanian, 2009; Shanahan, 2009). It is estimated that the GIT of healthy adults accommodates around 300-400 various cultivable species belonging to more than 190 genera (Holzapfel *et al*., 1998). Overall, there are between 1,000 to 1,150 prevalent bacterial species, and each individual person harbours at least 160 such species, which are predicted to be largely shared (Qin *et al*., 2010). The phylogenetic core of a healthy adult faecal microbiota consists of a set of five phyla: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria,* and *Verrucomicrobia* (Tap *et al*., 2009). The predominant genera in the gut microbiota, identified by molecular methods, include *Faecalibacterium, Ruminococcus, Eubacterium, Dorea, Bacteroides, Alistipes* and *Bifidobacterium* (Tap *et al*., 2009). Within the known GIT microbiota, only a few major groups dominate at levels around 10^{11}/g faeces, these are all strict anaerobes such as *Bacteroides, Eubacterium, Bifidobacterium* and *Peptostreptococcus* (Holzapfel *et al*., 1998). The GIT microbiota has many important functions in the human gut such as nutrient synthesis, digestion and absorption, immune stimulation and the control of pathogens. In both humans and mice, it has been reported that the onset of obesity is associated with changes in the relative abundance of the two dominant bacterial phyla in the gut, the *Bacteriodetes* and *Firmicutes* (Ley *et al*., 2005; Ley *et al*., 2006; Turnbaugh *et al*., 2009). Gut microbiota have been reported to promote obesity by increasing the

capacity of the host organism to extract energy in the form of calories from ingested food, this was displayed by transferring the gut microbiota from obese (*ob/ob*) mice to germ-free wild-type (WT) recipients, which led to an increase in fat mass in the recipients (Turnbaugh *et al*., 2006).

Toll-like receptor (TLR) 5 is a transmembrane protein that recognises bacterial flagellin and is highly expressed in the intestinal mucosa. In previous studies with mice genetically deficient in TLR5 (T5KO mice), it has been demonstrated that 10% of the mutant mice exhibited severe colitis and an extra 30% exhibited gross and/or histopathological evidence of colitis (Vijay-Kumar *et al*., 2007). The remaining 60% of the T5KO mice exhibited widely elevated proinflammatory gene expression but did not display the histopathologic features that define colitis, however, it was recorded that, by 4 weeks of age, these mice had body masses that were on average 15% higher than those of their WT littermates.

T5KO mice were reported to possess body masses that were 20% greater than those of WT mice, with an increased fat mass throughout their body, particularly in visceral fat as revealed by magnetic resonance imaging (MRI). The epididymal fat pads of T5KO mice were about twice as large as those in WT littermates at 20 weeks of age. This increase in fat mass was found to be associated with a substantial increase in serum levels of triglycerides and cholesterol, and also with an increase in blood pressure, features often observed with metabolic syndrome. Also it was reported in T5KO mice, a higher production of proinflammatory cytokines interferon-γ (IFN- γ) and interleukin-1β (IL-1 β) in T5KO adipose tissue versus WT adipose tissue, this would suggest that

increased adiposity may play a part in the attenuation of the low-grade chronic inflammation exhibited by the mutant mice.

After an overnight (15-hour) fast, T5KO mice displayed mild but statistically significant elevations in blood glucose compared to WT littermates. Furthermore, when T5KO mice were administered a bolus of glucose, they displayed impaired ability to restore blood glucose to baseline levels. Basal insulin levels on the other hand, were found to be significantly elevated in T5KO mice, as was the amount of insulin produced in response to glucose challenge. T5KO mice also exhibited a reduced response to exogenous insulin compared to WT mice, all these features are indicative of insulin resistance.

In this study undertaken by Vijay-Kumar et al. (2010), it was investigated if alterations in the gut microbiota as a loss of TLR5 promote the onset of metabolic syndrome in these mice. T5KO mice were administered broad-spectrum antibiotics upon weaning for 12 weeks. This treatment lowered gut bacterial load by 90% and led to the enlargement of the ceca. The decimation of the gut microbiota corrected T5KO metabolic syndrome relative to similarly treated WT mice. To determine whether the changes in the gut microbiota were a cause or consequence of the metabolic syndrome in T5KO mice, the T5KO microbiota was transplanted into WT germ-free mice, which, like newborn mice can be colonised by a diverse microbiota in a manner that preserves the complex composition of the transferred organisms (Turnbaugh *et al*., 2009). In principle, any gut dysbiosis present in the host would be transferred to the recipient (Garrett *et al*., 2007; Turnbaugh *et al*., 2006). It was reported that the transplanted T5KO microbiota conferred various aspects of the T5KO phenotype to the WT germ-free hosts, such as hyperphagia,

obesity, hyperglycemia, insulin resistance, colomegaly, and elevated levels of proinflammatory cytokines. These effects suggest that the changes in the gut microbiota seen in the T5KO mice are likely to be a predetermining aspect in the onset of metabolic syndrome in mice. From this study, it was concluded that the specific composition of microbiota to which individuals are first exposed may be an important means by which early environment exerts a lasting influence on metabolic phenotype (Vijay-Kumar *et al*., 2010). Also the excess caloric consumption promoting the currect epidemic of metabolic syndrome may be caused partly, by changes in host-microbiota interactions.

In newborns, high bifidobacterial counts are especially important in early infancy (Chierici *et al*., 2003). A stable microbiota, rich in *Bifidobacterium* species is usually established after the first weeks of life. However, in some infants the appearance of bifidobacteria is significantly delayed (Mitsuoka, 1992). In general, *Bifidobacterium* constitute 40-60% of the total faecal microbiota of a 2-week-old infant; however, in some formula-fed infants, bifidobacteria can be non-detectable, and can constitute 90% of total microbiota in some exclusively breast-fed infants (Harmsen *et al*., 2000a; Harmsen *et al*., 2000b). Breastfeeding and environmental factors influence gut colonisation (Marques *et al*., 2010). One of the main components of human milk are oligosaccharides (Thurl *et al*., 1996), which have been identified as a "bifidogenic" factor of human milk (Kunz and Rudloff, 1993; Newburg *et al*., 2000). Moro et al. (2002) reported that supplementation of a formula for term infants with a mixture of galacto- and fructooligosaccharides promoted the growth of *Bifidobacterium* and *Lactobacillus* species in the GIT and led to softer stools in a dose-dependent manner (Moro *et al*., 2002). In industralised countries there is often a low colonisation rate of bifidobacteria, even in breast-fed infants, this is

probably related to routine hygienic procedures (Hall *et al*., 1990; Lundequist *et al*., 1985).

Bifidobacteria predominate in newborns, and in breast-fed infants constitute up to 95% of the intestinal microbiota (Yoshioka *et al*., 1991), and 75% in formula-fed infants (Hadadji *et al*., 2005). Breast-fed infants generally harbour higher levels and a more diverse *Bifidobacterium* population compared to formula-fed infants (Roger *et al*., 2010; Roger & McCartney, 2010). In the adult GIT bifidobacteria form a mere 3% of the overall gut microbiota (Vaughan *et al*., 2005). During weaning, bifidobacterial numbers gradually decrease and *Bacteroides* and *Eubacterium* become predominant, but nevertheless *Bifidobacterium* are still a main and vital part of the adult intestinal microbiota. In Northern Europe, bifidobacterial content accounts for 4.4 ± 4.3% of the total faecal microbiota of adults (Lay, 2005), and *Bifidobacterium longum* subsp. *longum* is one of the ten most prevalent species found (Tap *et al*., 2009). In old age, bifidobacteria become reduced in number while *Clostridium* and other species increase (Mitsuoka, 1978). The overall faecal composition of an individual changes with advancing age, and in elderly subjects a decrease in the total number of bifidobacteria and a decline in species diversity has been detected. This may play a role in the susceptibility of these individuals to disease (Hopkins *et al*., 2001; Hopkins *et al*., 2002; Woodmansey *et al*., 2004).

In specific age groups, and in geographically distinct population groups, the dominant bifidobacterial species differ (Matsuki *et al*., 1999; Reuter, 2001). In an individual, the bifidobacterial community is often diverse and several bifidobacterial species and several strains of the same bifidobacterial species may colonise the GIT

simultaneously (Kimura *et al*., 1997; Mangin *et al*., 1999; McCartney *et al*., 1996; Satokari *et al*., 2001). In a study carried out by Silvi et al. (2003), LAB strains were isolated, using selective media from the faecal samples of elderly Italian subjects. In this study, it was reported that with regards to the bifidobacterial microbiota, the mean counts were more stable and on average higher than those of lactobacilli. These results were also reported by Hartemink, (1999) and Rowland, (1998). Furthermore, Silvi and co-workers, (2003) demonstrated with the identification of *Bifidobacterium* in each of the faecal samples, that there is a typical set of species for each person, with *B. longum* (7 people out of 12) being the most represented species (Silvi *et al*., 2003).

It was reported that only a limited number of bifidobacterial species such as *B. longum, B. adolescentis, B. breve, B. pseudocatenulatum* appeared to be dominant in the human intestinal and faecal samples investigated (Turroni *et al*., 2009), and may therefore be considered widespread *Bifidobacterium* species, on the other hand it was also discovered that certain other *Bifidobacterium* species seemed to be restricted to a particular ecological niche such as *B. bifidum* and *B. pseudolongum* (Turroni *et al*., 2009).

1.6 METABOLIC ACTIVITIES OF BIFIDOBACTERIA

1.6.1 Characteristics and effects of Conjugated linoleic acid isomers

Conjugated linoleic acid (CLA) is a natural component of ruminant milk and tissue fat, and is a mixture of positional and geometric conjugated isomers of the essential fatty acid linoleic acid. The major biologically active CLA isomers are *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) and *trans*-10, *cis*-12 CLA (*t*10, *c*12-CLA), (Ip *et al*., 1994; Park *et al*., 1999). About 80% of CLA found in dairy products is *c*9, *t*11-CLA. Its existence in ruminant fat is a consequence of the microbial biohydrogenation of dietary linoleic and linolenic acids to stearic acid in the rumen via the action of the enzyme linoleic acid isomerase (Kepler *et al*., 1966) or by the endogenous conversion of vaccenic acid to CLA via the action of the enzyme Δ9-desaturase (Corl *et al*., 2001; Griinari *et al*., 2000). Linoleic acid (LA) (C18:2n-6, precursor of n-6 series of fatty acids) and α-linolenic acid (C18:3n-3, precursor of n-3 series of fatty acids) are the only two fatty acids that cannot be synthesised by humans and thus are considered nutritionally essential (Nettleton, 1995). Mammalian cells can metabolise these fatty acids through a series of elongation and desaturation reactions, whereby LA is converted to arachidonic acid (C20:4n-6) and α-linolenic acid is metabolised to eicosapentaenoic acid (EPA) (C20:5n-3) by the enzymes D_6 desaturase, D_5 desaturase and elongase (Simopoulos, 2002). These highly unsaturated fatty acid metabolites have vital roles in cell membrane function and in the development and function of the brain and nervous system.

CLA has been linked with many physiological activities such as anti-adipogenic, anti-diabetogenic, anti-carcinogenic, and anti-atherosclerotic properties (reviewed in (Belury, 2002). CLA was originally described as an anti-carcinogenic agent in grilled

ground beef (Hayek *et al*., 1999). During both the initiation and promotion stages of carcinogenesis CLA has been shown to be anti-carcinogenic, and in inhibiting the development of mammary tumours, CLA has been reported to be more anti-carcinogenic than any other fatty acid tested (Ip *et al*., 1991). Activators of PPARγ have been shown to suppress colon cancer growth (Sarraf *et al*., 1998), and CLA is a ligand for the peroxisome PPARγ, and growth of colon cancer cells has been reported to be repressed in a dose-dependent fashion by CLA exposure (Kim & Park, 2003). Animal studies have shown that the anti-carcinogenic effects of CLA are observable at dosages of 0.5-1% (w:w) of the total diet (Park *et al*., 2004; Petrik *et al*., 2000). Pomegranate seed oil (PGO) from pomegranate seeds contains punicic acid, which is a form of conjugated linolenic acid (CLN). It was reported by Kohno et al. (2004), that PGO, rich in *c*9, *t*11, *c*13-CLN, significantly inhibited the development of azoxymethane (AOM)-induced colonic adenocarcinomas in male F344 rats, without any adverse effects. In this trial, different groups of rats were administered AOM+0.01% PGO, AOM+0.1% PGO, AOM+1% PGO or AOM alone as the control group. At all the dose levels of PGO tested, it was observed that there was a significant reduction of multiplicity of carcinomas in the colon of the rats compared with the control group (Kohno *et al*., 2004).

CLA has been shown to normalize impaired glucose tolerance and prevent or slow the development of hyperglycemia in the pre-diabetic Zucker fatty rat (Houseknecht *et al*., 1998). CLA has also been reported to reduce leptin in Zucker diabetic fatty rats (Belury & Vanden Heuvel, 1997), nondiabetic mice (Tsuboyama-Kasaoka *et al*., 2000), and humans with type 2 diabetes (Belury *et al*., 2003). Leptin is a hormone secreted by adipose tissue that regulates food intake so it may be significant to note that dietary CLA

reduces food intake in mice and rats (Belury & Kempa-Steczko, 1997; Park *et al*., 1997). The study performed by Belury et al. (2003), reported that lower body weights and serum leptin values in human subjects with type 2 diabetes were associated with *t*10, *c*12-CLA isomer accumulation in the plasma more so than the *c*9, *t*11-CLA isomer.

Previous studies have found that CLA positively influenced calcium and bone metabolism (Brownbill *et al*., 2005; Kelly & Cashman, 2004; Watkins & Seifert, 2000; Watkins *et al*., 1997), and Rahman et al. (2006) reported that CLA is a strong inhibitor of osteoclastogenesis, which increases during inflammatory-induced bone loss such as rheumatoid arthritis, periodontal disease, and postmenopausal osteoporosis (Rahman *et al*., 2003). CLA may have possible uses as a dietary therapeutic agent to treat bone diseases caused by chronic inflammatory conditions.

1.6.2 Bifidobacterial CLA production

CLA production by *Bifidobacterium* species was first reported by Coakley et al. (2003). It was observed that *B. breve* NCIMB 702258 was the most efficient strain for the conversion of LA to CLA. It converted 66% of LA to *c*9, *t*11-CLA and 6.21% of LA to *t*9, *t*11-CLA. Another strain *Bifidobacterium breve* NCIMB 8815 was also found to efficiently convert LA with 40% conversion to *c*9, *t*11-CLA and 2.87% conversion to *t*9, *t*11-CLA. It was reported by Coakley et al. (2003), that the maximum conversion of LA to *c*9, *t*11-CLA was associated with logarithmic and early stationary phase growth of the culture. The CLA isomer was primarily found in the cell supernatant fluid, while very small amounts were recovered in the cell pellet. Also it was recorded that the concentration of the *c*9, *t*11-CLA isomer decreased following 27 h of growth when *c*9,

*t*11-CLA was further converted to *t*9, *t*11-CLA. CLA production in bifidobacterial strains appears to vary from strain to strain as reported by Barrett et al. (2007). In this study, nine CLA-producing strains belonging to *B. longum* species were isolated, six strains belonging to *B. breve*, and a single strain each belonging to *B. infantis*, *B. dentium*, and *B. catenulatum.* The ability of these CLA producing strains to convert free LA to CLA varied from 76.65% conversion to 2.60% conversion. In the diverse population that was screened for CLA production in the study, it was recorded that one in four subjects harboured a CLA-producing *Bifidobacterium* strain. In this study the best source of CLA-producing bifidobacteria within a group, in terms of both conversion of linoleic acid to CLA and prevalence of CLA-producing, was the faecal material of elderly patients infected with the bacterium *Clostridium difficile.* Others have reported the isolation of CLA-producing bifidobacteria from the faecal material of neonates (Oh *et al*., 2003; Rosberg-Cody *et al*., 2004).

It was demonstrated by Hennessy et al. (2009), that some antioxidants, sugars and certain prebiotic compounds had a negative impact on *c*9, *t*11-CLA production by *B. breve* NCIMB 702258. The sodium salts of acetate, propionate and butyrate were some of the most effective compounds tested for increasing the production of *c*9, *t*11-CLA by the strain tested. This study revealed that yeast extract was one of the most effective prebiotic components for increasing *B. breve c*9, *t*11-CLA production. This would be expected as yeast extract is a plentiful source of amino acids, short peptides, B-vitamin complexes, carbon, nitrogen, minerals and trace ingredients all of which benefit the growth of bifidobacteria (Dave & Shah, 1998; Gomes *et al*., 1998; Klaver *et al*., 1993; Poch & Bezkorovainy, 1988; Poch & Bezkorovainy, 1991). It was reported by Oh et al.

(2003) that CLA production by two *Bifidobacterium* strains examined in their study, which were a *B. breve* strain and a *B. pseudocatenulatum* strain, with maximal production being in the early stationary phase of growth at 30 h. The *B. breve* and *B. pseudocatenulatum* cultures produced the *c*9, *t*11-CLA isomer, which accounted for 96% and 93% of their total CLA isomer products, respectively. The CLA production of the two cultures varied according to the growth stage of the two strains. CLA was found to be mainly present in the extracellular phase, i.e. in the growth medium; much less CLA was found to be intracellular when the cultures were tested for CLA production after fermentation. The cell pellets of each *Bifidobacterium* culture used in this study were washed with 50 mM Tris/HCL buffer (pH 7.5). It was reported that the washed cells when tested also produced CLA, which accumulated mainly as intracellular or cell-associated lipids (Blaut & Clavel, 2003).

It was reported by Coakley et al. (2006), that the *c*9, *t*11-CLA and *t*9, *t*11-CLA isomers produced by the isomerase activity of *B. breve* NCIMB 702258 exhibited antiproliferative activity against two different colon cancer cell lines (SW480 and HT-29 cancer cells). The *t*9, *t*11 isomer was shown to have a more potent effect than the *c*9, *t*11 isomer in both cell lines, with 10 µg/ml significantly reducing growth of SW480 cells after 48 h incubation, and after 4 days incubation the same concentration reduced growth by 43%. An incubation for 48 h with 20 µg/ml *c*9, *t*11-CLA, significantly reduced SW480 cell growth by 35%, whereas after 4 days incubation 10-20 µg/ml *c*9, *t*11-CLA significantly reduced growth by up to 65%. Also maximum inhibition of HT-29 cell growth was achieved with 56 µg/ml *c*9, *t*11-CLA and with 17 µg/ml *t*9, *t*11-CLA. The study by Coakley et al. (2009) reported that five strains of bifidobacteria, all of human

intestinal origin were capable of converting alpha-linolenic acid to conjugated linolenic acid (CLNA). This microbially produced CLNA exhibited inhibitory effects on the growth of SW480 colon cancer cells, *in vitro*. One of these products was identified as *c*9, *t*11, *c*15 C18:3-CLNA produced by *B. breve* NCIMB 702258. A concentration of 50µg/ml (180 µM) *c*9, *t*11, *c*15 C18:3-CLNA led to an 85% reduction in SW480 colon cancer cell numbers (Coakley *et al*., 2009).

1.6.3 Immunostimulation

Bifidobacteria are known to have immunodulatory effects on host mucosal cells, as seen in the human and animal studies in Table 1.2, except for one large human study which showed no statistically significant differences. The interaction of various *Bifidobacterium* species can have different effects on the host immune system as seen in Table 1.2; also different strains of the same species can interact with host mucosal cells producing varying immune responses. It is reported in some of the studies carried out, that bifidobacteria in combination with other bacterial species can have important immunodulatory effects on the host organism. From the studies listed in Table 1.2, bifidobacteria are generally reported to have a positive effect on the host immune system. The exact mechanisms of the immunodulatory activity of bifidobacteria and other intestinal bacteria are not fully known and presently are undergoing extensive scientific research.

1.6.4 Short Chain Fatty Acid production

Short chain fatty acids (SCFAs) are organic fatty acids of one to six carbon atoms long in the acyl chain, and are water soluble and readily absorbed in the body (McNeil *et al*., 1978). They are found naturally in foods such as fruits, vegetables and particularly, in milk fat (Newmark and Young, 1995). SCFAs are the main products of anaerobic breakdown of polysaccharide, oligosaccharide, and protein in the rumen and large intestine of plant-eating animals, where they can be found at millimolar concentrations (Cummings *et al*., 1987). Acetate (C2), propionate (C3) and butyrate (C4) are the major anions of SCFAs found in the lumen of the human large intestine (von Engelhardt *et al*.,

1989). Bifidobacteria metabolise non-digestible carbohydrates such as cellulose and starch, which are hexoses, through the "bifid shunt" catabolic pathway that uses fructose-6-phosphate phosphoketolase, producing phosphoenolpyruvate (PEP) which is degraded to pyruvate, the primary metabolite for SCFA production. Pyruvate is further metabolised by bifidobacteria to form acetyl-CoA, this then leads to the formation of acetate, lactate and ATP. This pathway produces 1.5 moles acetate for every mole of hexose that enters (Sela *et al*., 2008).

SCFA concentrations are highest in the proximal colon and are estimated to range from 70 to 140 mM (Cook, 1998), and decrease progressively towards the distal colon where they range from 20 to 70 mM (Roberfroid, 2005; Topping & Clifton, 2001). SCFAs are metabolised at three main locations in the body: colonocytes which utilise butyrate through β-oxidation as the main energy source; liver cells which metabolise butyrate and propionate for glucogenesis, also approximately 50 to 70% of acetate is absorbed by the liver; the third site are muscle cells that generate energy from the oxidation of residual acetate (Roberfroid, 2005).

Butyrate has been shown to be a major source of energy for colonic epithelial cells (Cummings, 1981; Cummings *et al*., 1987; Roediger *et al*., 1982). It has been reported to provide isolated colonocytes with around 60-70% of their energy requirements (Roediger & Millard, 1996) whilst also providing the body with 7-10% of its energy needs (Roy *et al*., 2006). Butyrate has also been found to cause differentiation of mammalian and carcinoma cells at low concentrations (Kruh, 1981; Tanaka *et al*., 1989). It is believed to prevent carcinogenic effects by mutagens at their active site, i.e. DNA (Smith, 1995). It has been observed in rats, that the supplementation of butyric acid

to a diet containing 20% margarine inhibited mammary tumour formation by 7, 12-dimethylbenz[a]anthracene. A study by Roediger, (1980), reported that butyrate uptake in colonic mucosal cells of patients with ulcerative colitis was reduced compared with normal controls, and postulated that a reduced utilisation of butyrate could be a predetermining factor in ulcerative colitis.

Propionate is largely absorbed by the liver, whereas acetate is metabolised by peripheral tissues during peripheral circulation (Wong *et al*., 2006). The study performed by Lankaputhra and Shah, (1998) detailed that all the strains of *Bifidobacterium* tested produced acetate. It was also reported that acetic and lactic acid accounted for > 90% of the organic acids produced. Acetate is the major SCFA produced in the colon, but departs the colon to be utilised by the liver, muscle and other peripheral tissues (Kien *et al*., 1992; Puchowicz *et al*., 1999). Acetate has been reported to provide the body with around 1.5-2 kcal/g (Topping & Clifton, 2001). It has also been reported to prevent DNA and cell damage to freshly isolated colon cells activated by hydrogen peroxide (Abrahamse *et al*., 1999). Acetate has been found to stimulate sodium absorption in the rat colon. The study undertaken by Argenzio and Whipp, (1979), reported that acetate transport into the cell in the perfused pig colon drives Na^+-H^+ exchange, as the secreted H^+ ion facilitates absorption of the unionised acid which is acetate. This leads to water being absorbed by the body, which could possibly have a benefit to patients suffering from various diarrhoeal conditions, as often these patients have lost a lot of water from their bodies.

Production of SCFAs in the colon might also directly influence ammonia absorption. A study undertaken by Visek, (1978), revealed that ammonia can induce changes in intestinal cells which can lead to tumour growth. In this study it was detailed

that the production of SCFAs in the colon directs nitrogen metabolism towards bacterial protein synthesis, this lowers luminal ammonia concentrations, and diminishes ammonia absorption, thereby reducing the risk of tumour growth.

The fermentation of different polysaccharides gives rise to different SCFA profiles. Acetate was found to be the main product of pectin and xylan breakdown, while large amounts of acetate and propionate were produced from arabinogalactan. In one study, butyrate was the only SCFA found to be produced in large amounts from starch (Englyst *et al*., 1987). The study performed by Jiang and Savaiano, (1997), showed that supplementation with *B. longum* B6 decreased lactose concentration during a three-day continuous culture at pH 6.7. This reduction in lactose concentration may be due to the increased production of acetate. This suggests that bifidobacteria may have the potential to help manage colitis by changing bacterial metabolism from lactate to SCFA formation.

1.6.5 Prebiotic utilisation

A prebiotic is defined as, "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health" (Gibson & Roberfroid, 1995). It has been observed that the growth and metabolic activity of bifidobacteria can be selectively promoted by prebiotics (Gibson & Roberfroid, 1995). Prebiotics have been shown to reduce the populations of non-beneficial bacteria by promoting microbial competition (Chow, 2002). Lactulose and galactooligosaccharides (GOS) are widely used in Europe, Japan and the United States as prebiotic carbohydrates (Tuohy *et al*., 2005). The first carbohydrate commercialised with recognised beneficial

effects on gut bifidobacteria was lactulose, a synthetic disaccharide created by the isomerisation of lactose in basic media (Aider & De Halleux, 2007; Mendez & Olano, 1979; Rycroft *et al*., 2001). GOS on the other hand, generally originates from enzymatic transgalactosylation of lactose (Mahoney, 1998; Panesar *et al*., 2006). Any fermentable dietary ingredient that passes undigested to the colon has the capability to act as a prebiotic. If anaerobic bacteria such as bifidobacteria, which have the metabolic capacity to degrade various oligo- and polysaccharides, did not colonise the distal region of the mammalian intestine, these dietary compounds would be lost (Vaughan *et al*., 2005). Bifidobacteria possibly acquired their specific ecological success from the ability to metabolise complex carbohydrates. As a consequence of this activity the host gains carbon and energy through the absorption of SCFAs, whereas bifidobacteria are afforded a vast amount of glycans and a safe anoxic environment. Bifidobacteria degrade oligo- and polysaccharides to monosaccharides and these are then metabolised to intermediates of the hexose fermentation pathway, which are then converted to SCFAs and other organic compounds (De Vries, 1969; Scardovi & Trovatelli, 1965).

Presently almost all prebiotics and all those manufactured commercially have been carbohydrates. These range from small sugar alcohols and disaccharides, to oligosaccharides and large polysaccharides (all with a number of sugar combinations and glycosidic linkages). Fructo-oligosaccharide (FOS) is the most commonly used commercial prebiotic, it is a mixture of oligosaccharides containing a varying number of fructose moieties connected by β (2-1) glycosidic bonds. *B. breve* UCC2003 genome analysis revealed a *fos operon*, encoding a putative permease, a conserved hypothetical

protein, and a β-fructofuranosidase all involved in the breakdown of short-chain FOS (Ryan *et al*., 2006).

In a study carried out by Cardelle-Cobas et al., (2009), it was observed that the four purified fractions derived from lactose and lactulose tested in their study acted as fermentable substrates for intestinal bacteria *in vitro*, leading to an increase in bifidobacterial numbers in human faecal cultures. A corresponding increase mainly in acetic acid and a moderate increase of propionic and butyric acid was also observed. It has been observed that carbohydrates with the degree of polymerisation (DP) = 3 showed the highest selectivity towards bifidobacteria (Kaneko *et al*., 1994; Kaplan & Hutkins, 2000).

It was also reported in the study undertaken by Carbelle-Cobas et al. (2009), that the GOS mixtures used, which were derived from lactose and lactulose contained trisaccharides with a high percentage of Gal β (1-6) and/or β (1-1) linkages, led to a similar or stronger bifidogenic effect than their precursor disaccharides and the commercial oligosaccharide Vivinal®-GOS (Cardelle-Cobas *et al*., 2009).

1.6.6 Health benefits of prebiotics involving *Bifidobacterium* species

It has been reported that a diet high in non-digestible fibres decreases body weight, fat mass and the severity of diabetes (Cani *et al*., 2005b; Cani *et al*., 2004; Cani *et al*., 2006a; Cani *et al*., 2006b; Cani *et al*., 2005c) . These dietary fibres increase the numbers of bacteria able to digest these fibres and thus supply extra energy to the host.

The study performed by Cani et al. (2008) investigated whether high-fat diet-induced diabetes could be improved in mice by selectively increasing bifidobacteria in

the gut microbiota. Mice fed a high-fat diet for two to four weeks were found to display a significant increase in plasma lipopolysaccharide (LPS). This endotoxemia was characterised as a "metabolic endotoxemia", since the LPS plasma concentrations were 10 to 50 times lower than those recorded during a septic shock (Mitaka, 2005). LPS is known to be a strong inducer of inflammatory response and is involved in the release of certain cytokines that are key factors triggering insulin resistance (Cani *et al*., 2008). It has been reported that high-fat diet feeding changes gut microbiota leading to an increase in the Gram negative to Gram positive bacterial ratio. The numbers of *Bifidobacterium* species were reported to be reduced, and these Gram positive bacteria have been found to reduce endotoxin levels in rodents and improve mucosal barrier function (Griffiths *et al*., 2004; Wang, 2004; Wang *et al*., 2006)

The prebiotic dietary fibre oligofructose [OFS] (Tuohy *et al*., 2005), was fed to specifically increase the number of bifidobacteria in the gut of high-fat diet treated mice. It was reported that among the different gut bacteria analysed, plasma LPS concentrations correlated negatively with *Bifidobacterium* species (Cani *et al*., 2007). OFS stimulates the release of a satiety-inducing gut hormone, GLP-1 (7-36) amide, and its precursor proglucagon mRNA in the proximal colon of rats (Cani *et al*., 2005a; Cani *et al*., 2004; Cani *et al*., 2005d). In mice fed a high-fat diet exhibiting a higher endotoxemia, this endotoxemia was completed abolished by feeding the prebiotic oligofructose. In the prebiotic treated mice, *Bifidobacterium* species significantly and positively correlated with improved glucose-tolerance, glucose-induced insulin-secretion, and normalised inflammatory tone (decreased endotoxemia, plasma and adipose tissue pro-inflammatory cytokines), (Cani *et al*., 2007). This study suggests that gut microbiota contributes to the

pathophysiological regulation of endotoxemia, and sets the tone of inflammation for the occurrence of diabetes/obesity. It would be therefore beneficial to develop specific methods to favour bifidobacteria growth and prevent the deleterious effect of high-fat diet-induced metabolic disease.

OFS promotes satiety following breakfast and dinner, and suppresses hunger and possible food consumption in subjects after dinner compared to placebo which was maltodextrin (DM) (Cani *et al*., 2006a). During OFS feeding it was reported that breakfast, lunch and total energy intake were moderately (5-10%), but significantly lower than those observed during the DM treatment. It was also reported that in rats, OFS supplementation decreased food intake – with effects on fat mass development, steatosis and hyperglycemia – through the promotion of intestinal synthesis and portal release of GLP-1 (7-36) amide (Cani *et al*., 2005b; Cani *et al*., 2004; Cani *et al*., 2005c) . GLP-1 (7-36) amide has been found to be a satiety hormone which causes weight loss in humans when delivered exogenously at levels in the range of physio to supraphysiologic doses (Flint *et al*., 1998; Flint *et al*., 2001; Verdich *et al*., 2001).

Prebiotic supplementation for 2 weeks is correlated with lowered subjective hunger (Cani *et al*., 2009), as was previously described by Cani et al. (2006a) Other studies involving animals have reported that changes in gut microflora after fermentable dietary carbohydrate ingestion decrease food intake, body weight, and fat mass development by activities correlated with the upregulation of endogenous GLP-1 and peptide YY production (Cani *et al*., 2005b; Cani *et al*., 2004; Cani *et al*., 2005c; Delzenne, 2005; Maurer *et al*., 2009; Zhou, 2006). The study by Cani et al. (2009) associated for the first time, the appetite regulation effects of prebiotics with increased

concentration responses of postprandial plasma gut peptide after a standardised meal in healthy subjects; this would support the role of the modulation of microbial activity or fermentation by prebiotics in the control of appetite sensations.

1.6.7 Exopolysaccharide production

The production of EPS has been reported for numerous strains of bifidobacteria including *B. pseudocatenulatum, B. longum, B. animalis* and *B. adolescentis* (Ruas-Madiedo *et al*., 2007). The physiological roles of these carbohydrate polymers have not yet been precisely established. Genetic studies and preliminary analyses carried out by Ruas-Madiedo et al. (2007) on the composition of EPS from human intestinal bifidobacteria revealed that they are heteropolysaccharides possessing glucose, galactose, and in many cases, rhamnose. Heteropolysaccharides are produced in much lower amounts than homopolysaccharides which would explain their low level of production in most intestinal bifidobacteria (Cerning *et al*., 1995; van Geel-Schutten *et al*., 1998). The study by Roberts et al. (1995) found that *B. longum* BB-79 produced the highest amount of EPS when lactose was used as the primary carbon source in liquid media. This represented a comparative increase of 200-300% of EPS yield over media containing sucrose, fructose or glucose. EPS was found to be produced in the log growth phase as after day 1 there was no significant increase in EPS production. EPS in bifidobacteria possibly play a role in cell recognition, adhesion to surfaces, and formation of biofilms to promote the colonisation of different ecosystems. They might also protect against phagocytosis, phage, and osmotic stress as seen in other bacteria (Looijesteijn *et al*., 2001; Whitfield *et al*., 1993).

In the study performed by Ruas-Madiedo et al. (2009), the production of EPS by *B. animalis* subsp. *lactis* in the presence of bile was detected. Two strains of *B. animalis* subsp. *lactis* were used in this study. One was the parental strain and the other a bile-adapted strain. Both strains appeared to have enhanced production of EPS in the presence of bile. These observations confirmed that bile promoted the synthesis of EPS in *B. animalis* subsp. *lactis*, possibly as a mechanism of protection against this toxic compound (Ruas-Madiedo *et al*., 2009). The study undertaken by Salazar et al. (2008) tested the abilities of some EPS produced by human intestinal bifidobacteria to act as fermentable substrates or prebiotics, and it was found that SCFA levels obtained in faecal cultures with the bifidobacterial EPS tested were similar to those obtained with the prebiotic inulin. Incubation of the intestinal microbiota with EPS led to an altered SCFA profile in faecal cultures, with an observed decrease in acetic acid and an increase in propionic acid levels, decreases or moderate increases in butyric acid levels, and a reduction in the acetic acid-to-propionic acid ratio through time. The highest decrease in the acetic acid-to-propionic acid ratio was found in four *B. longum* strains with EPS and with EPS C52 from *B. pseudocatenulatum*.

The EPS tested by Salazar et al. (2008), showed a bifidogenic effect with moderate increases in the levels of bifidobacteria during the faecal slurry culture incubation. The level of the effect depended on the EPS and the donor and was lower than that observed with glucose, being similar to that found with inulin. In the study performed by Salazar et al. (2009), it was observed that the EPS-producing strains of human origin had higher rhamnose content than strains of food origin examined previously by other authors. It was also found that in the EPS tested, the monosaccharides

galactose and glucose were universally present, and rhamnose in half of those tested. Also there were three major sugar components in EPS isolated from food environments, which were mannose, fucose, and N-acetyl-glucosamine. The high rhamnose content of EPS from *Bifidobacterium* has been linked to the protection against gastric ulcers induced in rats (Nagaoka *et al*., 1994).

1.6.8 Implications of *Bifidobacterium* species metabolism in the host

As bifidobacteria are naturally found in the human GIT, their presence and metabolic activities are considered integral to the maintenance of human health and well being, as bifidobacteria are found in the human GIT throughout life, present just days after birth (van der Werf & Venema, 2001). Bifidobacteria are found in the GIT in a reverse correlation to undesirable bacteria such as clostridia and *E. coli,* as observed from clinical feeding studies (Alvaro *et al*., 2007; Chen *et al*., 1999), which would suggest that bifidobacteria's metabolic activities have a role in preventing the growth in the GIT of these undesirable microorganisms. The utilization of non-digestible substrates by *Bifidobacterium,* such as complex carbohydrates like cellulose and starch provides the human host with a valuable energy source which would otherwise be lost from the body, unused as waste to the outside environment (Vaughan *et al*., 2005).

Bifidobacteria as an indigenous bacterium in many human hosts have developed a symbiotic relationship with their host as both the bacterium and the carrier benefit from the association. Bifidobacteria are provided with substrates and anaerobic conditions to support their growth, and the host benefits from the beneficial metabolic activities of the *Bifidobacterium* species that inhabit its GIT.

1.7 BIFIDOBACTERIA AS PROBIOTICS

Probiotics are described as such as 'live microorganisms, which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Bifidobacteria are used globally as probiotics in many food products such as yoghurt, milk, infant formula, cheese, and dietary supplements (Champagne *et al*., 2005; Charalampopoulos *et al*., 2002; Mattila-Sandholm *et al*., 2002; Phillips *et al*., 2006; Vinderola *et al*., 2000). As discussed above, *Bifidobacterium* can have positive influences on host immunostimulation; this ability has made bifidobacteria very attractive as probiotics.

1.7.1 Safety of *Bifidobacterium* species as probiotics

Bifidobacteria have a long history of safety as probiotics in fermented foods and dairy products, and the risk of infection from ingested bifidobacteria is very low (Gasser, 1994; Ouwehand *et al*., 2002). In a study undertaken by Abe et al. (2009) , it was discovered that the three bifidobacterial strains namely *Bifidobacterium longum* BB536, *Bifidobacterium breve* M-16V and *Bifidobacterium infantis* M-63, have no mucin degrading activity, and that *B. longum* BB536 has no translocation ability in the normal host. *B. longum* BB536 and *B. breve* M-16V are used as probiotics in many different foods and their continued use as such is safe. In Japan, *B. longum* BB536 is used as a functional ingredient in products designated as Food for Specified Health Uses (FOSHU) (Shortt, 1999).

Administration of *B. animalis subsp. lactis* and *B. bifidum* to mothers during the final month of pregnancy and infants during the first 6 months of life, most of whom

were at an advanced risk of atopic disease, was not associated with an increase in adverse events, which were defined as any untoward medical occurrence in a subject in the trial (Allen *et al*., 2010)

1.7.2 Survival of *Bifidobacterium* species in the GIT

An important property of any probiotic is the ability to survive passage through the GIT, as this affects the probiotic's ability to positively influence the host's indigenous microbiota. It can only be expected that the beneficial effects of probiotics can be seen when viable cells of these organisms are able to survive passage through the human stomach and digestive system and colonise the GIT (Kailasapathy & Chin, 2000). Colonisation of the GIT may be unnecessary but it has been suggested that colonisation is a highly desirable trait for an ingested probiotic organism (Klaenhammer, 1982; O'Sullivan *et al*., 1992). This would allow the ingested probiotic microorganism to maintain a sufficient level to influence its environment without regular replenishment (Kullen *et al*., 1997). Probiotic bifidobacteria are capable of conferring health benefits even though they might be briefly exposed to very acidic conditions after ingestion, and may experience a log-scale reduction of viability, there may still be a sufficient concentration of bifidobacteria in the GIT, depending on the dose (Jia *et al*., 2010). The study performed by Kullen et al. (1997), demonstrated that the *Bifidobacterium* strain tested in this study survived passage through the GIT; it became the predominant strain detected after 8 d of feeding. *B. animalis* subsp. *lactis* HN019 was shown to survive GI transit in a study carried out by Gopal et al. (2001). Live *B. animalis* subsp. *lactis* HN019 were recovered in the faeces of subjects consuming this strain. In certain subjects,

numbers as high as 12.5×10^8 CFU/g wet faeces were recovered, this revealed that the strain was able to reach the colon alive and proliferate.

In general, *Bifidobacterium* species isolated from the human GIT have been shown to be more sensitive to acid conditions than those from animal sources (Sanz, 2007). This is why strains of the species *B. animalis* are predominantly used in fermented probiotic products in Europe (Gueimonde *et al*., 2004). It is preferable to use human isolated *Bifidobacterium* species as probiotics for human consumption, as these species are found there naturally, and have adapted to the human GIT, and would therefore have a stronger possibility of colonising here. This is why the use of acid-stress treatments could further enable the use of human derived *Bifidobacterium* species as probiotics for human consumption, overcoming the technological obstacles posed by their low-intrinsic stress tolerance (Sanz, 2007). In the study carried out by Jia et al. (2010) , it was observed that strains of *B. bifidum*, *B. longum* and *B. longum* subsp. *infantis* were able to survive gastric conditions (pH 3) after 5 h, with counts greater than 2 log CFU/ml, which indicates that these strains are quite resistant to acidic conditions. These strains survived well in 0.45% (w/v) bile salt, with more than 4.5 log CFU/ml present after 10 h. The physiological bile salt concentration in the GIT of humans is estimated to be 0.3% to 0.4% w/v. There was an overall increase of bifidobacteria in the gut from 0.8% of total anaerobes to 16% of total bacterial counts, following feeding probiotic bifidobacterial strains to human subjects (Grmanova *et al*., 2010). The number of bifidobacteria decreased 7 days after administration and after 14 days were not detected, this showed that the bifidobacterial strains in this study didn't colonise the GIT.

1.7.3 Stability of *Bifidobacterium* species in probiotic supplements

An important property of a probiotic is its ability to survive in the carrier material, as this ensures that the probiotic bacteria remains viable and retains its metabolic activity so that it can function at its target site when consumed by the host (Holzapfel *et al*., 1998). In the study performed by Abe et al. (2009), it was observed that the probiotic *B. longum* and *B. breve* strains they tested were stable in Indonesian powdered formulas, as they detected counts of 1×10^7 CFU/g of bifidobacteria after 24 months storage at 30˚C which is presumed to be the ambient temperature in Indonesia. It has been observed that higher water activity leads to a lower survival rate (Higl *et al*., 2007; Schoug *et al*., 2006). The commercial formula had a significantly lower water activity than the powdered formula used in the strain comparison tests.

When *B. longum* was introduced to skimmed milk, the bacterium displayed a log-linear reduction over time when the pH value was low (pH 4.0, 4.25) but displayed shouldering and/or tailing as the pH value increased (Jayamanne & Adams, 2009). On the other hand, *B. animalis* subsp. *lactis* survived significantly longer, with survival measured in weeks rather than hours. The optimum survival temperature for *B. lactis* was over a range of temperatures from 4 to 8˚C. It survived longer at a low oxidation-reduction potential (E_h) and high pH values. These results indicate that *B. animalis* has a greater ability to withstand environmental stress conditions, such as high oxygen and acidity environments. This is why it has been widely used as a probiotic bacterium in many different foods.

1.7.4 Use of *Bifidobacterium* species to establish a healthy microbiota in preterm infants.

Before birth the infant GIT is sterile (Claud & Walker, 2001), and the rapid establishment of a healthy GIT microbiota in preterm infants is considered to be important for mucosal host defenses and in the prevention of some intestinal infections (Dai & Walker, 1999). Some studies listed in (Table 1.2) have been carried out to determine the effect of early oral supplementation with bifidobacterial probiotics on preterm infants. These studies have shown that early supplementation reduces the time for a bifidobacterial population to be established in the immature gut (Marques *et al*., 2010). This would be expected when large numbers of bacteria are ingested orally, which possibly offers some protective effects against GIT infection.

1.7.5 Use of *Bifidobacterium* species to treat infectious diarrhoea.

Infectious diarrhoea is a worldwide health problem. Among infants and children it remains a leading cause of illness and death in many developing countries (Thapar & Sanderson, 2004). Even in more developed countries, nosocomially-contracted diarrhoeal disease can significantly lengthen hospital stays and increase overall medical costs. The supplementation of probiotic bacteria such as bifidobacteria to infants can help to address this problem. Table 1.2 lists some human and animal studies undertaken involving bifidobacteria and their ability to treat infectious diarrhoea.

1.7.6 Improvement of lactose maldigestion using *Bifidobacterium* species

It has been reported that about two-thirds of the world's adult population suffers from lactose maldigestion (Vesa *et al*., 2000). Lactose maldigestion is caused by a reduction in lactase β-galactosidase (β-gal) activity in the small intestine after weaning. In lactose intolerant individuals, unhydrolysed lactose passes into the large intestine, where it is fermented by the native microbiota into gases such as hydrogen, methane, and carbon dioxide and SCFAs. The excessive gas production and the osmotic effects of excessive undigested lactose can lead to GI disturbances such as flatulence, abdominal pain, and diarrhoea (Jiang *et al*., 1996). This leads to the avoidance of milk and other dairy products by lactose intolerant people.

Bifidobacteria as a lactose-digesting culture has been used in studies using lactose-intolerant people to determine if it can alleviate the symptoms of lactose intolerance (Table 1.2). The effects reported in these studies are significant and generally regarded as scientifically sound, because the mechanism of action (digestion of lactose) is clear.

1.7.7 Use of *Bifidobacterium* species to reduce serum cholesterol levels

A high level of serum total cholesterol has been linked to an increased risk of cardiovascular disease (Taranto *et al*., 2004). Some studies involving the supplementation of bifidobacteria to reduce serum cholesterol have been undertaken (Table 1.2). One difficulty these studies have is obtaining an accurate baseline cholesterol measurement, as these levels can alternate. This is one reason why these studies generally do not show a significant effect of bifidobacteria supplementation in reducing serum cholesterol levels.

The exact mechanism by which bifidobacteria could reduce serum cholesterol levels remains unclear. The production of the enzyme bile salt hydrolase has been suggested as one possible mechanism (De Smet *et al*., 1998; St-Onge *et al*., 2000).

1.7.8 *Bifidobacterium* species and protection against infection

An important property of bifidobacteria as a probiotic bacterium is the ability to help prevent infection by pathogenic bacteria such as *E. coli*, *Salmonella* and *H. pylori*. These pathogenic bacteria if allowed to infect and colonise the GIT would cause detrimental effects for the host so the presence of bifidobacteria in the GIT is very important to prevent this occurring. Several mechanisms of protection have been proposed for bifidobacteria such as SCFA production, hydrogen peroxide or bacteriocins, competition for adhesion receptors or nutrients, anti-toxin action and stimulation of the immune system (Fooks & Gibson, 2002; Rakoff-Nahoum *et al*., 2004; Steer *et al*., 2000). Table 1.2 lists some studies carried out involving bifidobacteria and their potential protection against infection.

1.7.9 *Bifidobacterium* species and prevention of cancer

Cancers of the gut such as colorectal cancer which is the second most common cancer after lung cancer in men and breast cancer in women in Europe (Boyle & Langman, 2000), could be prevented by the administration of a probiotic such as bifidobacteria. The studies listed in Table 1.2 involving bifidobacterial anti-carcinogenic effects are mainly animal studies, which provide some evidence of the anti-carcinogenic effects of bifidobacterial supplementation. Most of these studies used bifidobacterial

supplementation in combination with a prebiotic to reduce the risk of cancer-inducing cells in the model tested. At present, there are no human studies with conclusive evidence displaying a protective effect of this type of supplementation.

1.8 CONCLUSIONS AND FUTURE IMPLICATIONS

As bifidobacterial genomics expands, our understanding of this probiotic bacterium increases. One important aspect of bifidobacteria that needs further investigation is their interactions as an indigenous bacterium within a host organism. Furthermore, the interactions of bifidobacteria introduced as new probiotic species with the resident bifidobacteria in the GIT also needs further research. This would determine if more symbiotic associations can be influenced by the introduction of probiotic bifidobacteria, with a view to maintaining an overall balance of healthy bacteria in the human host. As more *Bifidobacterium* species are being sequenced, it is possible that new insights into bifidobacterial interactions within the human host will be revealed. The identification of the genes involved in the production of bioactive fatty acids by bifidobacteria is important, as these are currently unknown. The identification of these genes would make it possible for their manipulation in order to produce increased concentrations of these bioactive fatty acids. Bifidobacterial strains producing large quantities of these fatty acids could be given as a probiotic supplement, to improve gut health or prevent certain metabolic disorders. The increased understanding of bifidobacteria as a probiotic bacterium would be useful in developing new bifidobacterial probiotics, with specific benefits to the recipient. The probiotic carrier would need to provide the most suitable conditions like optimum pH, water activity, and redox potential to allow the *Bifidobacterium* strain contained within to survive and colonise more efficiently within the host's GIT. This would optimise the benefit of the probiotic's interactions and metabolic activities within the host. The colonisation of probiotic *Bifidobacterium* species within the human GIT would negate the need for continual supplementation of

that probiotic species. As the 'Functional Foods' market expands, bifidobacteria can have a major role to play in the development of new probiotic foods specifically for human health.

With the increasing need to prove the health conferring benefits of probiotics, it is vital that the beneficial capacities of bifidobacteria are proven in the laboratory. Bifidobacterial genomics provides us with the opportunity to achieve these aims. There is also a need for the proper selection and characterisation of bifidobacterial strains intended for probiotic use. As the incidence of metabolic diseases such as cancer and obesity increasing worldwide, it is possible that new probiotic species of *Bifidobacterium* can help alleviate some of these conditions within the human host.

As the understanding of bifidobacterial substrate utilization capabilities expands, developing more suitable substrates such as new prebiotics to help establish and/or maintain the colonization of bifidobacteria in the human host will be possible. New substrates could be added to the human diet to help maintain and promote the growth of resident and new probiotic *Bifidobacterium* species within the human GIT. It is likely that future research into bifidobacteria will focus on the development of appropriate conditions within a probiotic to maintain the bifidobacterial strain or strains contained within, so that they will arrive in the GIT in an optimum state to influence the human host. It is also likely that new metabolic activities of bifidobacteria will be revealed from bifidobacterial genomics, which will help in the design of new probiotics to specifically treat certain diseases in humans.

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Table 1.1 – Habitats and references of the species of the genus *Bifidobacterium*

Species	**Habitat first identified from**	**Reference**
B. actinocoloniiforme	Bumblebee intestine	Killer et al. (2010a)
B. adolescentis	Faeces of human adult, bovine rumen, sewage and human vagina	Reuter (1963)
B. angulatum	Sewage, faeces of human adult	Scardovi & Crociani (1974)
B. animalis subsp. *animalis*	Animal faeces	Scardovi & Trovatelli (1974)
subsp. *lactis*	Yoghurt	Meile et al. (1997)
B. asteroides	Honeybee intestine	Scardovi & Trovatelli (1969)
B. bifidum	Faeces of human adult, infant, suckling calf and human vagina	Orla-Jensen (1924)
B. bohemicum	Bumblebee intestine	Killer et al. (2010a)
B. boum	Bovine rumen, faeces of piglet	Scardovi et al. (1979)
B. bombi	Bumblebee intestine	Killer et al. (2009b)
Bombiscardovia coagulans	Bumblebee intestine	Killer et al. (2010b)
B. breve	Faeces of infant and suckling calf, human vagina and sewage	Reuter (1963)
B. catenulatum	Faeces of infant and human adult and sewage	Scardovi & Crociani (1974)
B. choerinum	Faeces of piglet and sewage	Scardovi et al. (1979)
B. coryneforme	Honeybee intestine	Biavati et al. (1982)
B. crudilactis	Raw milk and raw milk cheeses	Delcenserie et al. 2007
B. cuniculi	Faeces of rabbit	Scardovi et al. (1979)
B. dentium	Human dental caries and oral cavity; faeces of human adult, abscess and appendix	Scardovi & Crociani (1974)
B. gallicum	Human faeces	Lauer (1990)
B. gallinarum	Chicken caecum	Watabe et al. (1983)
B. indicum	Honeybee intestine	Scardovi & Trovatelli (1969)
B. infantis	Faeces of infant and suckling calf	Reuter (1963)
B. longum subsp. *longum*	Faeces of human adult	Reuter (1963)
subsp. *infantis*	Faeces of infant	Reuter (1963)
subsp. *suis*	Faeces of piglet	Matteuzzi et al. (1971)
B. magnum	Faeces of rabbit	Scardovi & Zani (1974)
B. merycicum	Bovine rumen	Biavati & Mattarelli (1991)
B. minimum	Sewage	Biavati et al. (1982)
B. mongoliense	Fermented mare's milk product from Mongolia	Watanabe et al. (2009)

B. pseudocatenulatum	Faeces of infant and suckling calf and sewage	Scardovi et al. (1979)
B. pseudolongum subsp. *pseudolongum*	Faeces of pig, chicken, bull, calf, rat and guinea pig	Yaeshima et al. (1992) ex Mitsuoka (1969)
subsp. globosum	Faeces of piglet, suckling calf, rat, rabbit and lamb; sewage; bovine rumen	ex Biavati et al. (1982)
B. psychraerophilum	Pig faeces	Simpson et al. (2003)
B. pullorum	Faeces of chicken	Trovatelli et al. (1974)
B. ruminantium	Bovine rumen	Biavati & Mattarelli (1991)
B. saeculare	Faeces of rabbit	Biavati et al. (1991)
B. scardovii	Human blood	Hoyles et al. (2002)
B. stercoris	Human faeces	Kim et al. (2010)
B. subtile	Sewage	Biavati et al. (1982)
B. thermacidophilum subsp. *theracidophilum*	Waste water from anaerobic digester	Dong et al. (2000)
B. thermophilum subsp. porcinum	Faeces of piglet	Mitsuoka (1969)
B. tsurumiense	Hamster dental plaque	Okamoto et al. (2008)

Table 1.2 Summary of some clinical studies identifying bifidobacterial potential health

Health benefit	Study Summary	Reference
Establishment of a healthy microbiota in preterm infants	Large human study (69 preterm infants) showing that supplementation with *B. animalis* subsp. *lactis* Bb-12 increased the number of bifidobacteria, while reducing the numbers of enterobacteria and clostridia.	Mohan et al. (2006)
	Medium-sized human study (33 preterm infants per group) which observed that supplementation with *B. breve* reduced ($P<0.05$) faecal butyric levels. This was only observed in the subgroup of infants weighing >2,500 g.	Wang et al. (2007)
	Large human study (66 preterm infants) which reported that supplementation with *B. breve* decreased ($P<0.05$) aspirated air volume from the stomach and improved weight gain.	Kitajima et al. (1997)
	Large human study (145 preterm infants) which were fed a probiotic mixture (*B. longum* subsp. *infantis*, *Streptococcus thermophilus*, and *B. bifidus*) reduced both the severity and incidence of necrotising enterocolitis in those fed the probiotic ($P=0.005$).	Bin-Nun et al. (2005)
Immunostimulation	Small human study (7 healthy children) showing that feeding a powdered cow milk-based follow-up formula for children containing *B. animalis* subsp. *lactis* Bb-12 significantly increased total faecal IgA and anti-poliovirus IgA.	Fukushima et al. (1998)
	Small human study (8 patients with ulcerative colitis per group) supplemented with *B. longum*, inulin, and fructooligosaccharides showed no significant change in symptom scores but showed some decrease ($P<0.05$) in the expression of genes encoding human proinflammatory cytokines.	Furrie et al. (2005)
	Small mouse study (10 per group) showing that supplementation with *B. animalis* subsp. *lactis* Bb-12 led to significantly higher levels of total IgA and anti-β-lactoglobulin IgA in faecal extracts and milk of lactating dams compared to lactating dams not fed *B. lactis* Bb-12.	Fukushima et al. (1999)

	Large mouse study (30 per group) showing that supplementation with *B. longum* promoted dendritic cell maturation in Peyer's Patches, up-regulated IL-10, IL-12, interferon-γ (IFN-γ) mRNA and the interferon-γ/IL-4 ratio in intestinal mucosa, increased IFN-γ gene expression and raised immunoglobulin secretion in cultured peripheral blood mononuclear cells.	Dong et al. (2010)
	Small mouse study (6 per group) which reported that supplementation with *Bifidobacterium* significantly suppressed the skewed Th2 response and increased the number of regulatory T cells in allergic mice.	Zhang et al. (2010)
	Large rat study (156 rats) showing that supplementation with *B. longum* significantly inhibited colon, small intestine, and liver tumour incidences in male rats ($P>0.05$). In female rats supplementation of *B. longum* also decreased mammary carcinogenesis to 50% and liver carcinogenesis to 27% of those observed in rats fed the control diet, but these differences were not statistically significant ($P>0.05$).	Reddy & Rivenson, (1993)
	Two small mice studies (8 or 9 per group using BALB/c and SCID mice) and a small pig study (16 weanling pigs) showing that supplementation with *Bifidobacterium breve* NCIMB 702258 and linoleic acid significantly increased liver *c*9, *t*11 CLA in both mice ($P<0.001$) and pigs ($P<0.05$). This led to the reduced ($P<0.05$) production of the inflammatory metabolites interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-8, and IL-12, combined with an increase in the regulatory cytokine, IL-10. Also significantly ($P<0.001$) higher concentrations of eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA) were found in adipose tissue of *B. breve* supplemented mice compared to the control fed mice.	Wall et al. (2009)
	Large mouse study (18 or 20 per group) showing that supplementation with *B. animalis* subsp. *lactis* ($P<0.01$), *Lactobacillus rhamnosus* ($P<0.005$), or *Lactobacillus acidophilus* ($P<0.005$) enhanced phagocytic cell function, and production of IFN-γ and increased immune mediated resistance to challenge to *Salmonella typhimurium*.	Gill et al. (2000)
	Large human study (5 groups of 13 to 15 subjects supplemented with different amounts of *Lactobacillus paracasei* and *B. animalis* subsp. *lactis*) which showed no statistically significant differences in cytokine levels.	Christensen et al. (2006)

	Medium-sized human study (12 or 13 elderly subjects per group) which showed some increase (P<0.05*)* in the anti-inflammatory cytokine IFN-α and in phagocytic activity after 6 weeks of supplementation with *B. animalis* subsp. *lactis*.	Arunachalam et al. (2000)
Cholesterol reduction	Small human study (18 or 11 women per group) showing an increase in high-density lipoprotein (HDL) levels but no reduction in total cholesterol (P=0.001) in subjects fed yoghurt with *Lactobacillus acidophilus* and *B. longum*.	Kiessling et al. (2002)
	Small rat study (5 per group) showing that supplementation with *B. breve* protected (P<0.05) against the increase of serum total cholesterol and lower density lipoprotein (LDL) cholesterol compared to that of a high cholesterol di*et al*one.	Rhee et al. (2002)
	Small rat study (7 per group) and medium-sized human study (32 adult males) showing that supplementation with milk containing *B. longum* and starter cultures led to lowering (P>0.05) of serum levels of total cholesterol, LDL cholesterol, and triglycerides, with no change in HDL cholesterol levels compared to the control.	Xiao et al. (2003)
Lactose intolerance	Small human study (15 lactose intolerant people) showing some reduction (P<0.05) in breath hydrogen after supplementation with *B. longum*.	He et al. (2007)
	Small human study (11 Chinese lactose intolerant individuals) supplemented with *B. animalis* subsp. *lactis* and yoghurt cultures showing some reduction (P<0.05) in symptom scores.	
Prevention of infectious diarrhoea	Large human study (55 infants) showing a small (P=0.035) protective effect of *B. bifidum* and *Streptococcus thermophilus* supplementation, seen as reduced shedding of rotavirus (P=0.025).	Saavedra et al. (1994)
	Small human study (10 infants per group) showing that diarrhoea caused by rotavirus infection does not affect *B. animalis* subsp. *lactis* Bb12 adherence (P=0.84). Also *B. animalis* subsp. *lactis* Bb12 adherence was increased (P=0.018) during diarrhoea when used in combination with *Lactobacillus rhamnosus* GG.	Juntunen et al. (2000)
	Large human study (46 or 44 infants per group) showing a non-statistically protective effect of *B. animalis* subsp. *lactis*.	Choraqui et al. (2004)
	Large human study (69 elderly patients per group) which showed a non-statistically protective of *L. acidophilus* and *B. bifidum* supplementation against *Clostridium difficile* associated diarrhoea.	Plummer et al. (2004)

	Small piglet study (8 or 9 per group) which showed a moderate, statistically significant (P<0.01) protective effect of *B. animalis* subsp. *lactis* against rotaviral and *Escherichia coli* associated diarrhoea and a higher titre of anti-rotaviral antibodies in the faeces.	Shu et al. (2001)
	Large mouse study (29 to 41 per group) showing a statitically significant (P<0.001) protective effect against rotaviral diarrhoea of *B. bifidum* and *B. longum* subsp. *infantis* supplementation.	Qiao et al. (2002)
	Large mouse study (52 to 111 per group) which showed a small, statistically significant (P<0.05) protective effect of *B. bifidum* supplementation, with reduced rotaviral shedding (P=0.025).	Duffy et al. (1994)
Prevention of cancer	Large rat study (30 per group) which showed a significant reduction (P<0.001) in carcinogen-induced colonic neoplasms after supplementation with both *B. animalis* subsp. *lactis* in combination with resistant starch. No reduction was observed with *B. animalis* subsp. *lactis* alone.	Le Leu et al. (2010)
	Large rat study (4 groups of 15) which showed some reduction (P<0.05) in carcinogen-induced aberrant crypt foci following supplementation with *B. longum* and a significant decrease (P<0.001) after supplementation of *B. longum* in combination with inulin.	Rowland et al. (1998)
	Large human study (4 groups of 18 to 22 polypectomised or colon cancer patients) which showed that there was some improvement (P<0.05) only in polypectomised patients in epithelial barrier function and cell toxicity after supplementation with *B. animalis* subsp. *lactis*, *L. rhamnosus*, and inulin.	Rafter et al. (2007)
	Medium-size mouse study (12 per group) which showed some reduction (P<0.01) in occurrence of tumours when heat-killed *B. longum* subsp. *infantis* cells were injected into mice along with tumour cells, along with a significant increase (P<0.001) in the number of mice which were cured of tumours.	Sekine et al. (1985)
Prevention of infection	Small mouse study (7 or 8 per group) which showed that supplementation with milk containing a *B. breve* or a *Bifdobacterium catenulatum* strain significantly inhibited (P<0.01) the production of Shiga toxin by Shiga toxin producing *Escherichia coli* 0157:H7 in the intestine of infected mice.	Asahara et al. (2004)
	Large mouse study (30 per group) showing that supplementation with *B. longum* one month after *E. coli* infection led to disappearance of *E. coli* from the lung, kineys, spleen, and liver of germfree mice.	Romond et al. (1997)

	Small mouse study (9 per group) showing that supplementation with *Bifidobacterium thermacidophilum* attenuated intestinal injuries due to *E. coli* 0157:H7 infection, and significantly increased (P<0.01) anti-*E. coli* O157:H7 IgA titers and IgG+IgM response (P<0.01) 2 weeks after *E. coli* 0157:H7 infection.	Gagnon et al. (2006)
	Mouse study showing that supplementation with *B. longum* led to significantly (P=0.05) higher survival of mice infected with *Salmonella* Typhimurium compared to the control group.	Silva et al. (2004)
	Large human study (11 or 59 subjetcs infected with *Helicobacter pylori* per group) showing that 6 week supplementation with a yoghurt containing a *L. acidophilus* strain and *B. animalis* subsp. l*actis* Bb12 significantly (P<0.0001) decreased the urease activity of *H. pylori*. Also *B. animalis* subsp. *lactis* had an inhibitory effect on *H. pylori* growth in some patient samples *in vitro.*	Wang et al. (2004)

Adapted from Lee and O'Sullivan, (2010).

CHAPTER 2

Intraspecies diversity in *Bifidobacterium longum* with respect to genomic relatedness and microbial metabolite production.

2.1 ABSTRACT

Many potentially probiotic traits have been identified in *Bifidobacterium* species of which EPS production, CLA production and lantibiotic production were examined in this study. The differentiation of the bifidobacterial strains into subspecies using amplified ribosomal DNA restriction analysis was also performed. 38 bifidobacterial strains, obtained from infants and adults, were assessed *in vitro* for the selected probiotic traits using a combined phenotypic and molecular approach. Of the 38 strains tested, 27 strains were obtained from breast fed infants and one strain from a formula plus prebiotic fed infant. In addition, 10 CLA producing strains from a previous study (Barrett *et al.*, 2007) obtained from healthy infants, healthy adults and a *C. difficile* positive patient were used. Differentiation of the 38 strains into subspecies indicated that 34 were designated *B. longum* subsp. *longum* and four *B. longum* subsp. *infantis*.

The results of the screening analysis revealed that of the 38 *B. longum* strains tested, 15 *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain displayed an EPS phenotype in mMRS with 7% (w/v) lactose agar and broth. Of these, 24 *B. longum* subsp. *longum* and two *B. longum* subsp. *infantis* strains displayed a positive PCR product using specific primers for a previously identified pGTF of a bifidobacterial EPS operon (Audy *et al*., 2010). Nine *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain converted free linoleic acid to *cis*-9, *trans*-11 (*c*9, *t*11) CLA as reported by Barrett et al. (2007). With regard to bacteriocin production, lantibiotic production was not confirmed by the methods employed in this study, however, PCR analysis using primers specific to a previously identified lantibiotic structural gene *lanA* (Lee *et al*., 2011)

indicated the presence of the *lanA* gene in five *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain.

B. longum DPC 6315 exhibited an EPS phenotype and converted free linoleic acid to *c*9, *t*11 CLA *in vitro* and whilst it did not produce a detectable bacteriocin by the methods employed in this study, it contained the EPS priming glycosyltranferase gene *wblE* and lantibiotic prepeptide *lanA* gene.

2.2 INTRODUCTION

Since bifidobacteria were first isolated from the faeces of breast fed infants (Tissier, 1900), they have come to be regarded as one of the most important bacterial groups associated with human health. Bifidobacteria are widely used probiotics, defined as 'live microorganisms, which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). Bifidobacteria have a long history of safe use in fermented milks and dairy products, and the risk of infection from ingested bifidobacteria is minimal (Gasser, 1994; Ouwehand *et al.*, 2002).

Strains of *Bifidobacterium longum* have been reported in many human, animal and *in vitro* studies to possess probiotic effects such as immune stimulation (Wu *et al*., 2010), lowering of serum cholesterol (Xiao *et al*., 2003), alleviation of lactose maldigestion (Jiang *et al.*, 1996), prevention of infectious diarrhoea (Qiao *et al*., 2002) and anti-carcinogenic properties (Coakley *et al.*, 2003). Potential probiotic traits that have been reported for different strains of *B. longum* include the production of an EPS (Salazar *et al.*, 2008), production of CLA (Barrett *et al.*, 2007) and lantibiotic production (Lee *et al.*, 2011).

The production of EPS has been reported for numerous strains of bifidobacteria including *B. longum, B. adolescentis, B. animalis* and *B. pseudocatenulatum* (Ruas-Madiedo *et al*., 2007). It is generally accepted that EPS produced by bifidobacteria are heteropolysaccharides consisting of repeating units of polysaccharides and non-carbohydrate units, such as phosphate, acetyl and glycerol. An EPS operon was found in *Bifidobacterium longum* subsp. *longum* CRC 002 (Audy *et al.*, 2010) containing all the genes necessary for EPS assembly and biosynthesis, such as the *wblE* gene which plays

an important role in the initial stages of EPS biosynthesis. The *wblE* gene codes for the predicted pGTF and the C-terminal domain of this glycosyltransferase catalyses the transfer of a sugar-1-phosphate to a lipophilic carrier molecule anchored in the cellular membrane, this being the first step for the assembly of the repeating unit that builds many heteropolysaccharides (Audy *et al.*, 2010). EPS isolated from bifidobacteria have been demonstrated to exert prebiotic effects on other bifidobacteria and other bacterial groups (Salazar *et al*., 2008), positive immunological effects (Wu *et al*., 2010) and antimicrobial activity against pathogenic organisms (Wu *et al*., 2010).

In the study performed by Fanning et al. (2012), it was demonstrated that naïve splenocytes isolated from mice and stimulated with *B. breve* UCC2003 EPS producing cells had significantly ($P<0.01$) lower levels of proinflammatory cytokines IFN-γ, TNF-α and IL-2 compared to naïve splenocytes stimulated with *B. breve* UCC2003 deficient EPS producing cells (Fanning *et al*., 2012). It was also shown in the study of Fanning et al. (2012) that the presence of *B. breve* UCC2003 EPS producing cells significantly ($P<0.05$) reduced the colonisation levels of the gut pathogen *Citrobacter rodentium* in BALB/c mice compared to mice fed *B. breve* UCC2003 deficient EPS producing cells as early as 24 h and up to day 14 post infection.

CLA is a natural component of ruminant milk and tissue fat, and is a mixture of positional and geometric conjugated isomers of the essential fatty acid linoleic acid. The major biologically active CLA isomers are *cis*-9, *trans*-11 CLA (*c*9, *t*11-CLA) and *trans*-10, *cis*-12-CLA (*t*10, *c*12-CLA) (Ip, 1994; Park, 1999), which have been linked with many physiological activities such as anti-adipogenic, anti-diabetogenic, anti-carcinogenic, and anti-atherosclerotic activities (reviewed in (Belury, 2002)). CLA

production by bifidobacteria was first reported by Coakley et al. (2003), and in this study *c*9, *t*11-CLA was the most common isomer produced. CLA production by bifidobacteria has been reported to differ from strain to strain (Barrett *et al*., 2007), and in a study performed by Hennessy et al. (2009) some antioxidants, sugars and certain prebiotic compounds present in the growth medium were reported to enhance the production of *c*9, *t*11-CLA by *B. breve* NCIMB 702258.

Lantibiotics (lanthionine-containing antibiotics) are class I bacteriocins which are ribosomally synthesized small peptides, characterized by post-translational modification of primary residues into modified residues such as lanthionine (Ala-S-Ala) and β-methylanthionine Abu-S-Ala) (Chatterjee *et al.,* 2005). They have generally been reported to possess a broad inhibition spectrum of activity against other gram positive bacteria. Nisin produced by some strains of *Lactococcus lactis* was the first lantibiotic to be characterised and was first recognised as a non-acid inhibitor by Rogers in 1928 (Rogers, 1928). Nisin is now approved for use as a food preservative in approximately 50 countries due to its wide range of applications (Delves-Broughton, 1990). Lantibiotics are divided into type A or type B, depending on the topology of their ring structure and their biological activities (Jung, 1991). It is widely understood that type A lantibiotics disrupt the integrity of the cell membrane leading to pore formation, loss of ions and solutes and eventually cell death. A common mechanism is the dissipation of the proton motive force of sensitive cells (See review Guinane *et al.,* 2005 thesis). Unlike the pore-forming type A lantibiotics, type B peptides have no net charge, are hydrophobic in nature and do not form pores in the cell membrane. Mersacidin (Brötz *et al.,* 1997) and actagardine (Brötz

et al., 1997), both type B lantibiotics have been shown to inhibit cell wall synthesis, at the level of transglycosylation in target organisms (Brötz *et al*., 1997).

The first bifidobacterial lantibiotic was isolated from *B. longum* subsp. *longum* DJO10A (Lee *et al*., 2008). The lantibiotic produced by this *B. longum* strain was encoded by a characteristic lantibiotic operon, including the lantibiotic prepeptide or lantibiotic encoding gene *lanA*, and other genes encoding production, modification and regulatory functions (Lee *et al*., 2008). The *lanA* structural gene encodes a ribosomally synthesised precursor prepeptide which contains a leader sequence at the N-terminus, which is ultimately cleaved, and a propeptide at the C-terminus (Lee *et al*., 2011). This lantibiotic displayed a broad spectrum of inhibition against some closely related bacterial strains and also some potentially pathogenic strains (Lee *et al*., 2011). Lantibiotics can confer a selective advantage on the producer organism by inhibiting the growth of other organisms found in the same habitat. Lantibiotics have the potential to be used as natural food preservatives due to their high stability and broad inhibition spectrum (Chen & Hoover, 2003; Deegan *et al.*, 2006).

The aim of this study was to assess a range of *B. longum* strains, primarily of infant intestinal origin, for their ability to exhibit potentially probiotic traits *in vitro*: namely EPS, CLA and lantibiotic production. The differentiation of these 38 bifidobacterial strains into subspecies *longum* and *infantis* was also performed.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains and culture conditions.

38 *B. longum* strains, all of human intestinal origin, were used in this study, (Table 2.1). The strains abbreviated with DPC were isolated in the study of Barrett et al. (2007). Strains numbered 1 to 61 were obtained from the stool of breast fed infants and strain numbered 74 was obtained from a prebiotic formula fed infant (Barrett *et al.*, unpublished results). Strains were cultured on modified deMan Rogosa Sharpe (mMRS) (Difco Laboratories, Detroit, MI) medium supplemented with 0.05% (w/v) cysteine hydrochloride (98% pure; Sigma, St. Louis, MO) and 1.5% (w/v) agar (Oxoid, Hampshire, UK). Strains were cultured on mMRS medium supplemented with 0.05% (w/v) cysteine hydrochloride, 1.5% (w/v) agar (Oxoid) with 7% (w/v) lactose, and in mMRS with 7% (w/v) lactose broths to detect the EPS phenotype. Agar plates and broths were incubated anaerobically (anaerobic jars with Anaerocult® A gas packs [Merck, Darmstadt, Germany]) at 37°C for 72 h.

2.3.2 Nucleic acid extraction and manipulation.

Chromosomal DNA preparations were isolated from stationary phase cultures (~48 h) of the *B. longum* strains as outlined by Vincent et al. 1998 (Vincent *et al.,* 1998). DNA was quantified using a Thermo Scientific nanodrop 1000 (Mason Technology, Ireland). DNA was quantified using a Thermo Scientific nanodrop 1000 (Mason Technology, Ireland).

2.3.3 Species and subspecies-specific identification by amplified ribosomal DNA restriction analysis (ARDRA).

The differentiation of the *B. longum* strains into subspecies *longum* and subspecies *infantis* was performed by ARDRA analysis using the restriction enzyme Sau3AI and visualised by UV spectrophotometry following a previously described method (Šrůtková *et al*., 2011).

2.3.4 PFGE analysis.

High-molecular-weight chromosomal DNA was isolated from stationary-phase cultures as previously described (Hoffman & Winston, 1987). PFGE of all *B. longum* strains was undertaken as previously described (Simpson *et al.*, 2003) with the following modifications: Restriction enzyme XbaI (New England Biolabs) was used to cleave chromosomal DNA, and the fragments were separated using contour-clamped homogeneous electric field CHEF-DR III pulsed-field system (Bio-Rad Laboratories). Fragments were resolved with a linear ramp pulse time of 1 to 15 sec for 18 h at 6V/cm in a running buffer containing 0.5 × Tris base-borate-EDTA maintained at 12°C. DNA fragment sizes were estimated by comparison with low range PFG marker ranging in size from 2.03 kb to 194.0 kb (New England Biolabs) and DNA-macro-restriction profiles were visualised using the Alpha Imaging system.

The DNA macro-restriction profiles were stored as tagged image file format files and imported into Bionumerics software (Version 6.5, Applied Maths, Kortrijk, Belgium). The Dice coefficient of similarity was calculated and the unweighted pair group method with arithmetic averages (UPGMA) was used for cluster analysis with

optimisation and position tolerance set at 1.5%. A cut-off at 90% similarity of the Dice co-efficient was used to indicate identical PFGE patterns.

2.3.5 EPS Screening.

The loop touch test (Ruas-Madiedo & de los Reyes-Gavilan, 2005) was used to detect strains with an EPS phenotype on agar plates. For the detection of the EPS phenotype in mMRS with 7% (w/v) lactose broth, strains were incubated for 72 h in 30 ml mMRS with 7% (w/v) lactose broth. The mMRS with 7% (w/v) lactose broth was centrifuged at 3,500 × *g* for 10 min before analysis.

2.3.6 EPS isolation and quantification.

EPS was isolated from the 16 EPS positive phenotypic strains grown in mMRS with 7% (w/v) lactose broth for 72 h at 37°C following the isolation method of Mårtensson et al. (2002) with the following modifications. The pH of the samples were adjusted to pH 6.2 with 4M NaOH followed by overnight hydrolysis using 0.2 mg/ml proteinase K (Sigma-Aldrich, Wicklow, Ireland) at 37°C. To terminate the reaction, the mixture was heated at 90°C for 10 min and centrifuged at 4,000 × *g* for 30 min (Sorvall®LegendRT, Thermo Scientific, Loughborough, UK). The supernatant was collected and precipitated with 4 volumes of chilled ethanol and agitated (100 rpm) overnight at 4°C. To recover the precipitate, the mixture was centrifuged at 4,500 × *g* for 30 min. The pellet was dissolved in 10 ml of sterile deionised water and dialysed (molecular mass cut-off of 12, 000 Da) against deionised water for 3 d at 4°C (with two daily washing steps).

The colorimetric phenol-sulphuric method was performed to estimate the EPS content with glucose as standard (Dubois, 1956). The concentrations of EPS were determined by subtracting the total amount of glucose detected in unfermented culture medium (which was used as a blank) from total amount of glucose detected in the inoculated fermentation medium.

2.3.7 CLA screening.

Microbial conversion of linoleic acid to CLA was assessed using a previously described rapid CLA screening method (Barrett *et al*., 2007).

2.3.8 Screening of *wblE* and *lanA* genes by PCR analysis.

Primers specific to the *wblE* gene from *B. longum* subsp. *longum* CRC 002 (Audy *et al.*, 2010) EPS operon were designed and PCR was used to detect its presence in the strains used in this study. These primers were wblE F (5'-GGGTGTGGTCGCTCTATGTT-3') and wblE R (5'-GTCAGCCACCATAACGGTCT-3'). A second set of hybrid primers G-Bact-a-F-36 and G-Bact-a-R-27 which were previously developed by Provencher et al. (2003) were also used to detect the presence of the pGTF gene in the 38 *B. longum* strains.

To detect the presence of a previously described lantibiotic encoding *lanA* gene, primers F-lanA forward primer (5'-GTCCATTGTCGGTGAATCCT-3') and R-lanA reverse primer (5'-AGTTCCCACAGGATGCAAGT-3') were used for PCR amplification of the *lanA* structural gene (Lee *et al*., 2011).

2.3.9 Screening for lantibiotic presence and production.

The bacteriocin-producing capabilities of strain was determined using cell-free supernatants in a modified agar well diffusion assay described previously (McAuliffe *et al*., 1998) and using an overlay agar bioassay for lantibiotic production previously described in Lee et al. (2008). Indicator strains used included *B. breve* DPC 702258, *Bifidobacterium breve* DPC 6330 and each of the 38 *B. longum* strains were used as indicator strains to examine any zones of inhibition from the supernatants of the other 38 strains.

2.4 RESULTS

38 bifidobacterial strains were obtained from a number of different human sources which included both infants and adults as mentioned above. It was then necessary to classify these bifidobacterial isolates into subspecies. These *B. longum* strains were then tested for a number of potentially probiotic traits which included EPS, CLA and lantibiotic production.

2.4.1 Phylogenetic analysis.

ARDRA analysis was used to distinguish between the subsp. *longum* and subsp. *infantis* as previously described (Šrůtková *et al*., 2011). 38 strains were analysed and it was revealed that 34 belonged to *B. longum* subsp. *longum* and four to *B. longum* subsp. *infantis* (Table 2.1).

PFGE was also used to distinguish the strains using a method previously described by Simpson et al. (2003) and this analysis revealed that each of the 38 strains were distinct from each other, as each strain possessed a distinct PFGE profile (Fig. 2.1) and (Fig. 2.2). Certain distinct PFGE macro-restriction patterns appear to be highly conserved among the *B. longum* strains examined in this study, such as those at ~110 kb which was observed in 26 (68.4%) strains (Fig. 2.1) and (Fig. 2.2), at ~120 kb and at ~194 kb in 25 (65.8%) strains, at ~23.1 kb present in 21 (55.3%) and at ~9.42 kb in 19 (50%) strains of the 38 *B. longum* strains (Fig. 2.1) and (Fig. 2.2). PFGE macro-restriction patterns present in less than half of the *B. longum* strains were those at ~145.5 kb which was present in 18 (47.4%) strains, at ~97 kb and at ~48.5 kb present in 17 (44.7%) of strains and (Fig. 2.1) and (Fig. 2.2).

A dendogram generated based on computer comparisons of the DNA macro-restriction profiles of the 38 *B. longum* strains examined in this study revealed that all strains shared 40% or more genetic similarity. Indeed, some of the *B. longum* subsp. *longum* strains shared a very high percentage of similarity. Two such strains were 47 and 49, which shared approximately 80% genomic similarity (Fig. 2.3), these strains shared 12 macro-restriction patterns between their PFGE profiles (Fig. 2.2). Strain 56 shared approximately 70% genetic similarity with the two strains 47 and 49 (Fig. 2.3), sharing 9 macro-restriction patterns between their PFGE profiles (Fig. 2.2). Strains 17 and 19 shared approximately 75% genetic similarity (Fig. 2.3), sharing 9 macro-restriction patterns. Strains 1 and 9 shared approximately 68% genetic similarity with one another (Fig. 2.3), sharing 9 macro-restriction patterns. There was approximately 65% genetic similarity between the pairs of strains DPC 6315 and DPC 6322 (Fig. 2.3) (sharing 9 macro-restriction patterns) (Fig. 2.1), strains 28 and 58 (Fig. 2.3) (sharing 8 macro-restriction patterns) (Fig. 2.2) and the strains DPC 6321 and 13 (Fig. 2.3) (sharing 8 macro-restriction patterns) (Fig. 2.2).

2.4.2 Screening for probiotic traits: EPS production.

The loop touch test (Ruas-Madiedo & de los Reyes-Gavilán, 2005) and centrifugation (3,500 *g* × 10 min) of strains grown for 72 h in mMRS with 7% (w/v) lactose were used to detect strains with an EPS phenotype on agar plates and in broth respectively.

Of the 38 strains tested, 15 *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain possessed the EPS phenotype (Table 2.2). *B. longum* subsp. *longum* DPC

6315 and the *B. longum* subsp. *infantis* strain numbered 1, produced the highest amount of EPS of the *B. longum* strains tested when grown for 72 hours in mMRS with 7% (w/v) lactose broth in this study, with *B. longum* DPC 6315 producing 234.8 (± 5.4) mg/l and strain 1 producing 188 (± 4.2) mg/l (Table 2.2). EPS levels produced by the *B. longum* strains possessing the EPS positive phenotype in this study displayed a wide range from 23.8 (± 2.7) mg/l to 234.8 (± 5.4) mg/l (Table 2.2).

2.4.3 CLA production.

Using a rapid CLA screening method previously described in the study (Barrett *et al*., 2007), nine *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain converted free linoleic acid to *cis*-9, *trans*-11 (*c*9, *t*11) CLA as reported previously in the study Barrett et al. (2007). *B. longum* DPC 6315 was found to be the strain with the highest percentage conversion of linoleic acid to CLA of 60.1 ± 5.1% (Table 2.3). None of the remaining 28 *B. longum* strains were found to convert free linoleic acid to CLA (Table 2.3).

2.4.4 PCR analysis – *wblE* and *lanA* genes.

Two sets of primers specific to the *wblE* (pGTF) gene designed in previous studies were used to detect the presence of this EPS coding gene in the strains using PCR analysis. The *wblE* gene was found to be present in 24 *B. longum* subsp. *longum* and two *B. longum* subsp. *infantis* strains (Table 2.2). Interestingly two of the *B. longum* subsp. *longum* strains tested, numbered 52 and 53 displayed an EPS positive phenotype but did not possess the *wblE* gene (Table 2.2). Overall, 14 of the *B. longum* strains possessed the

EPS phenotype and gave a positive PCR result for the *wblE* gene, while 11 of the strains possessed only the *wblE* gene but did not display the EPS positive phenotype by the method used in this study (Table 2.2).

PCR analysis using previously described primers designed to amplify the *lanA* structural gene (Lee *et al*., 2011) revealed that 6 of the 38 *B. longum* strains were found to give a positive PCR result for the *lanA* gene (Table 2.4) using this specific primer set. These strains were *B. longum* DPC 6315, and *B. longum* subsp. *longum* strains numbered 3, 9, 28 and 36 and the *B. longum* subsp. *infantis* strain numbered 6 (Table 2.4).

2.4.5 Lantibiotic production.

The bacteriocin-producing capabilities of the strains was determined using cell-free supernatants in a modified agar well diffusion assay described previously (McAuliffe *et al*., 1998) and using an overlay agar bioassay for lantibiotic production previously described in Lee et al. (2008).

The modified agar well diffusion assays and overlay agar bioassays did not reveal any inhibitory action of the supernatants taken from the 38 *B. longum* strains on the indicator strains used, *B. breve* DPC 702258, *B. breve* DPC 6330 and the *B. longum* strains examined in this study (Table 2.4).

2.5 DISCUSSION

This study identified the subspecies' differentiation and certain probiotic traits of 38 *B. longum* strains obtained from human infant and adult intestines. Of the 38 strains, 34 were found to be *B. longum* subsp. *longum* and just four were typed as *B. longum* subsp. *infantis*.

PFGE analysis demonstrated that all the 38 *B. longum* strains were distinct from each other. However, the 38 *B. longum* strains did appear to share a high level of genetic similarity as found from dendogram analysis with all the strains sharing 40% or higher genetic similarity, and some strains such as 47 and 49 sharing approximately 80% genetic similarity. Certain DNA macro-restriction fragments appear to be found in a high proportion of the 38 *B. longum* strains such as those at ~110 kb, ~120 kb, ~194kb, ~23.1 and at ~9.42 kb. These patterns would appear to be highly conserved parts of the genomes of the *B. longum* strains examined in this study.

There were no noticeable differences reported in the probiotic traits identified in the *B. longum* subsp. *longum* and the four *B. longum* subsp. *infantis* strains. Of the 38 *B. longum* strains tested, *B. longum* DPC 6315 was found to possess an EPS phenotype, and produce the highest amount of EPS (234.8 ± 5.4 mg/l) in mMRS with 7% (w/v) lactose broth. The study performed by Barrett et al. (2007) revealed that it produced the highest percentage conversion of linoleic acid to CLA (60.1 ± 5.1%) of the *B. longum* strains tested. PCR analysis revealed that it also possessed the *wblE* gene of the EPS operon (Audy *et al.*, 2010) and *lanA* gene of the lantibiotic operon (Lee *et al*., 2011).

The presence of the *lanA* gene would suggest that *B. longum* DPC 6315 has the capability of producing a lantibiotic which has already been identified in *B. longum*

DJO10A (Lee *et al.*, 2008). This lantibiotic was purified and characterised in the study of Lee et al. (2011) and was found to inhibit both gram positive and gram negative bacterial strains including several bifidobacteria strains such as those from the species *longum, adolescentis, breve, bifidus* and *animalis*, two *Lactococcal lactis* strains, one *Streptococcus thermophilus* strain, one *Micrococcus luteus* strain, several potentially pathogenic strains such as *Enterococcus faecalis* LG110, a *Clostridium perfringens* strain, a *C. difficile* strain, a *Staphylococcus epidermidis* strain, a *Staphylococcus aureus* strain, a *Bacillus subtilis* strain, an *Escherichia coli* DH5a strain, a *Serratia marcescens* strain and a *Proteus vulgaris* strain. In their study, no production of the lantibiotic could be detected on agar, unless an inducer such as the purified lantibiotic itself was added to the broth (Lee *et al*., 2011). This effect was also seen in the streptin lantibiotic produced by *Streptococcus pyogenes* (Wescombe & Tagg, 2003).

No activity of the potential purified lantibiotic produced by *B. longum* DPC 6315 was observed in this study using well diffusion assays against indicator strains. This could be due to one or all of the genes essential for lantibiotic production being switched off in *B. longum* DPC 6315 or that the level of the lantibiotic produced by the strain was too low to detect under the conditions used in this study. It is possible that this lantibiotic could still have an effect *in vivo* as there is more competition for nutrients and binding sites from other gut bacteria in close proximity to the producer.

Overall, the results of this study reveal that a high percentage of the *B. longum* strains displayed a ropy phenotype after growth in mMRS with 7% (w/v) lactose broth and agar. The amount of EPS produced by the *B. longum* strains examined in this study varied widely with the lowest amount (23.8 ± 2.7 mg/l) being produced by *B. longum*

DPC 6321 and the highest amount (234.8 ± 5.4 mg/l) being produced by *B. longum* DPC 6315. EPS production appears to be a feature of *B. longum* growth; it possibly helps the bacterial cells to adhere to the mucosal cell walls and to resist environmental stresses in the GIT, conferring a select advantage over non-EPS producing organisms present in the gastrointestinal tract. In the strain *Bifidobacterium breve* UCC2003, the production of cell surface EPS provided stress tolerance to both acid (pH 5.0) and bile stress (0.3%) and promoted *in vivo* persistence but not initial colonisation in BALB/c mice compared to EPS-deficient variants of *B. breve* UCC2003 (Fanning *et al*., 2012). The study performed by Fanning et al. (2012) demonstrated that mice fed bacterial cells of *B. breve* UCC2003 strains deficient in EPS production had significantly (P<0.05-0.001) lower bacterial numbers compared to mice fed *B. breve* UCC2003 EPS producing cells. The bacteria were fed initially for 3 days and this result was seen from day 9 up to day 31 of the trial. On day 31, the viable counts of the *B. breve* UCC2003 strains deficient in EPS production were significantly (P<0.05) reduced within the murine caeca and colons compared to those of mice fed *B. breve* UCC2003 EPS-producing strains (Fanning *et al*., 2012). This would suggest that the production of EPS in *B. breve* UCC2003 is enhancing the strain's ability to persist in the murine intestine.

Interestingly, two of the *B. longum* subsp. *longum* strains tested, (numbered 52 and 53) displayed an EPS phenotype, but not a gtf phenotype but there are potentially many other genes from other bacteria and possibly bifidobacteria that code for polysaccharides, producing different EPSs. CLA production appears to be less common than EPS production in *B. longum* strains, as only ten of the 38 tested strains converted linoleic acid to CLA. With regard to bacteriocin production, the presence of the *lanA* gene was

detected in 6 of the 38 tested strains which would suggest this is a less common feature of *B. longum* strains.

As *B. longum* DPC 6315 was originally isolated from adult faeces, and has been found to survive passage through the murine intestine (Wall *et al.*, unpublished results), it has the ability to survive passage through the gastrointestinal tract which is a prerequisite for any probiotic bacterial strain. The production of EPS, CLA and the potential production of a lantibiotic by *B. longum* DPC 6315 make it an exciting multifacitated bacterial strain for future possible applications in a range of probiotic studies.

2.6 ACKNOWLEDGEMENT

Primers specific to the *wblE* gene in *B. longum* subsp. *longum* CRC 002 and primers specific to *lanA* gene in *B. longum* DJO10A were designed by Caitriona Guinane, Teagasc Food Research Centre Moorepark, Fermoy, Co. Cork, Ireland.

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Table 2.1 Strain, source, subspecies differentiation and origin of 38 *B. longum* strains used in this study.

Strain	Source	Subspecies	Origin
DPC 6315	G[1]	*longum*	Barrett et al. (2007)
DPC 6316	G[1]	*longum*	Barrett et al. (2007)
DPC 6317	A	*longum*	Barrett et al. (2007)
DPC 6318	C	*longum*	Barrett et al. (2007)
DPC 6319	I	*infantis*	Barrett et al. (2007)
DPC 6320	J	*longum*	Barrett et al. (2007)
DPC 6321	A	*longum*	Barrett et al. (2007)
DPC 6322	H	*longum*	Barrett et al. (2007)
DPC 6323	B[2]	*longum*	Barrett et al. (2007)
DPC 6324	B[2]	*longum*	Barrett et al. (2007)
1	D[3]	*longum*	Barrett et al. (2014)
3	D[3]	*longum*	Barrett et al. (2014)
6	F	*infantis*	Barrett et al. (2014)
9	D[4]	*longum*	Barrett et al. (2014)
10	D[4]	*infantis*	Barrett et al. (2014)
13	D[4]	*longum*	Barrett et al. (2014)
17	E	*longum*	Barrett et al. (2014)
18	F[5]	*longum*	Barrett et al. (2014)
19	F[5]	*longum*	Barrett et al. (2014)
21	F[5]	*longum*	Barrett et al. (2014)
22	F	*longum*	Barrett et al. (2014)
26	F	*longum*	Barrett et al. (2014)
28	D	*longum*	Barrett et al. (2014)
31	F	*infantis*	Barrett et al. (2014)
36	F	*longum*	Barrett et al. (2014)
38	D	*longum*	Barrett et al. (2014)
40	F	*longum*	Barrett et al. (2014)
43	F	*longum*	Barrett et al. (2014)
45	F	*longum*	Barrett et al. (2014)
47	E	*longum*	Barrett et al. (2014)
49	F	*longum*	Barrett et al. (2014)
52	D	*longum*	Barrett et al. (2014)
53	E	*longum*	Barrett et al. (2014)
56	D	*longum*	Barrett et al. (2014)
58	D	*longum*	Barrett et al. (2014)
59	E	*longum*	Barrett et al. (2014)
61	F	*longum*	Barrett et al. (2014)
74	D	*longum*	Barrett et al. (2014)

Legend

A=Healthy 3 day old baby. B=Healthy 4 day old baby. C=Healthy 5 day old baby. D=Healthy 1 week old baby. E=Healthy 4 week old baby. F=Healthy 27 week old baby. G=Healthy 25 year old adult. H=Healthy 31 year old adult. I=Healthy 37 year old adult. J=64 year old *C. difficile* positive patient. [1]Strains with matching superscript numbers indicate they originate from the same baby.

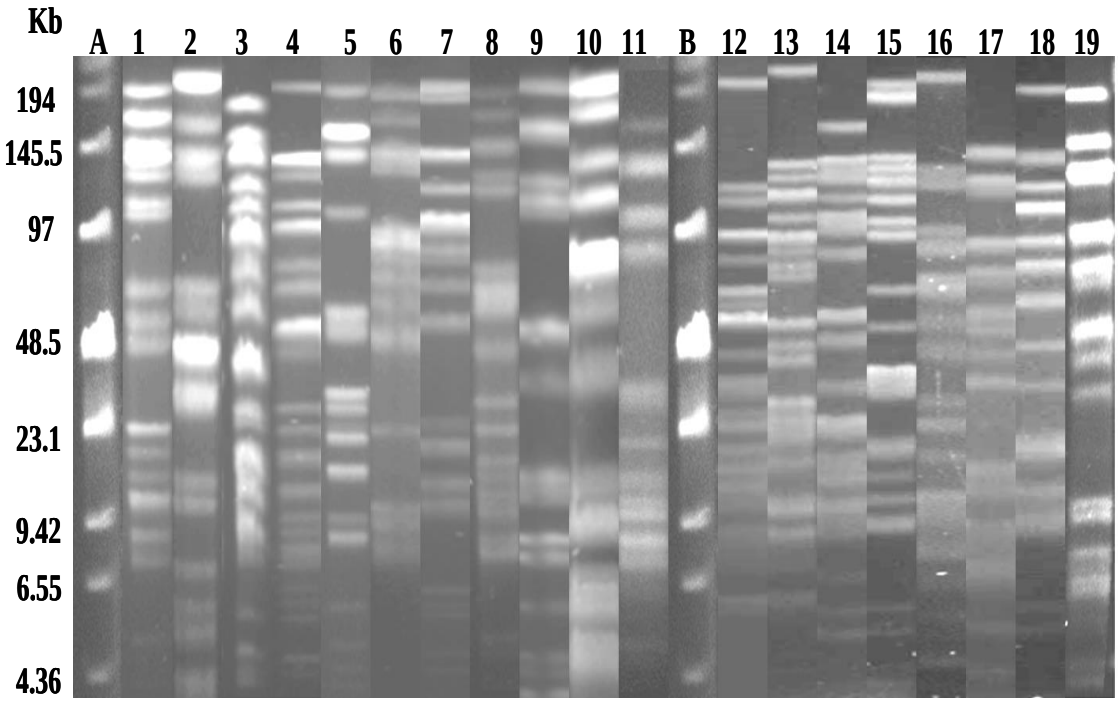


Figure 2.1 *B. longum* PFGE macro-restriction patterns following genomic DNA digestion with restriction enzyme XbaI.

Lanes A, B = Low range PFG marker. Lane 1 – DPC 6315, Lane 2 – DPC 6316, Lane 3 – DPC 6317 – Lane 4 – DPC 6318, Lane 5 – DPC 6319, Lane 6 – DPC 6320, Lane 7 – DPC 6321, Lane 8 – DPC 6322, Lane 9 – DPC 6323, Lane 10 – DPC 6324, Lane 11 – Strain 1, Lane 12 – Strain 3, Lane 13 – Strain 6, Lane 14 – Strain 9, Lane 15 – Strain 10, Lane 16 - Strain 13, Lane 17 – Strain 17, Lane 18 – Strain 18, Lane 19 – Strain 19.

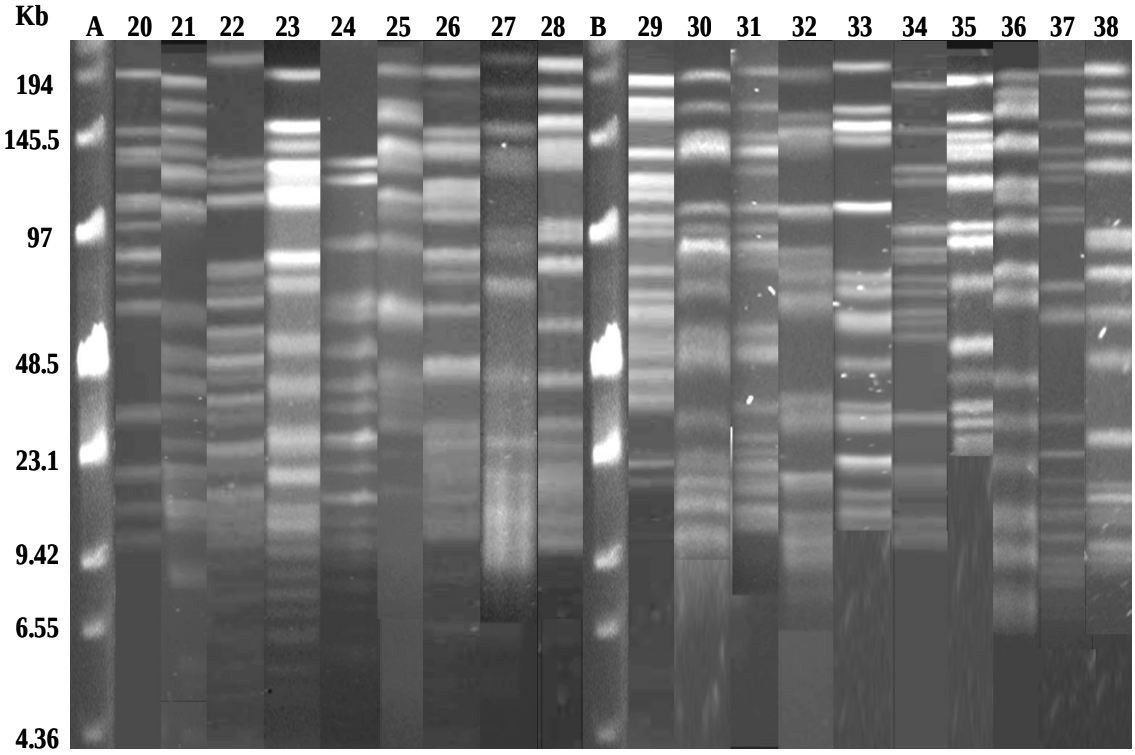
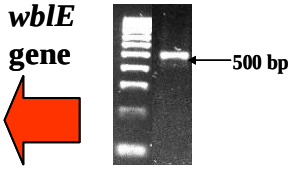


Figure 2.2 *B. longum* PFGE macro-restriction patterns following genomic DNA digestion with restriction enzyme XbaI.

Lanes A, B = Low range PFG marker. Lane 20 – Strain 21, Lane 21 – Strain 22, Lane 22 – Strain 26, Lane 23 – Strain 28, Lane 24 – Strain 31, Lane 25 – Strain 36, Lane 26 – Strain 38, Lane 27 – Strain 40, Lane 28 – Strain 43, Lane 29 – Strain 45, Lane 30 – Strain 47, Lane 31 – Strain 49, Lane 32 – Strain 52, Lane 33 – Strain 53, Lane 34 – Strain 56, Lane 35 – Strain 58, Lane 36 – Strain 59, Lane 37 – Strain 61, Lane 38 – Strain 74.

Table 2.2 Strain, presence of EPS phenotype, presence of *wblE* gene and amount of EPS (mg/l) produced by *B. longum* strains possessing either positive EPS phenotype or presence of *wblE* gene examined in this study.

Strain	Presence of EPS phenotype.	Presence of *wblE* gene. 	Amount of EPS produced (in mMRS with 7% lactose (mg/l).
B. longum subsp. *longum* DPC 6315	+	+	234.8 ± 5.4
B. longum subsp. *longum* DPC 6316	-	+	N.D.
B. longum subsp. *longum* DPC 6317	-	+	N.D.
B. longum subsp. *longum* DPC 6318	+	+	70 ± 4.9
B. longum subsp. *longum* DPC 6320	-	+	N.D.
B. longum subsp. *longum* DPC 6321	+	+	23.8 ± 2.7
B. longum subsp. *longum* DPC 6324	-	+	N.D.
B. longum subsp. *longum* 1	+	+	188 ± 4.2
B. longum subsp. *longum* 9	+	+	123.5 ± 3.8
B. longum subsp. *infantis* 10	+	+	71.7 ± 1.6
B. longum subsp. *longum* 13	-	+	N.D.
B. longum subsp. *longum* 18	-	+	N.D.
B. longum subsp. *longum* 19	-	+	N.D.
B. longum subsp. *longum* 21	+	+	66.8 ± 3.1
B. longum subsp. *longum* 22	-	+	N.D.
B. longum subsp. *longum* 26	+	+	113.8 ± 2.5
B. longum subsp. *longum* 28	+	+	90.8 ± 3.6
B. longum subsp. *infantis* 31	-	+	N.D.
B. longum subsp. *longum* 36	-	+	N.D.
B. longum subsp. *longum* 38	+	+	64.1 ± 1.3
B. longum subsp. *longum* 40	-	+	N.D.
B. longum subsp. *longum* 52	+	-	109.1 ± 3.2
B. longum subsp. *longum* 53	+	-	68 ± 4. 1
B. longum subsp. *longum* 56	+	+	55.4 ± 2.3
B. longum subsp. *longum* 58	+	+	70.1 ± 1.9
B. longum subsp. *longum* 59	+	+	32.7 ± 2.4
B. longum subsp. *longum* 61	+	+	102.5 ± 3.7
B. longum subsp. *longum* 74	-	+	N.D.

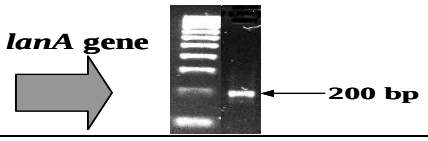
Legend: Presence of EPS phenotype: + depicts ropy phenotype in mMRS with 7% lactose. – depicts ropy phenotype not present in mMRS with 7% lactose. Presence of *wblE* gene: + depicts presence of *wblE* gene (DNA band present at ~500 bp). - depicts *wblE* gene not present (no DNA band present at ~500 bp). N.D. = Not detected

Table 2.3 Amount of *c*9, *t*11 CLA conversion (%) for each of the 38 *B. longum* strains examined in this study using a rapid CLA screening method previously described by Barrett et al. (2007).

Strain	Amount of *c*9, *t*11 CLA conversion (%)
B. longum subsp. *longum* DPC 6315	60.1 ± 5.1
B. longum subsp. *longum* DPC 6316	4 ± 2.6
B. longum subsp. *longum* DPC 6317	20.2 ± 3.8
B. longum subsp. *longum* DPC 6318	18.7 ± 0.3
B. longum subsp. *infantis* DPC 6319	3.7 ± 0.4
B. longum subsp. *longum* DPC 6320	53.1 ± 2.5
B. longum subsp. *longum* DPC 6321	38.5 ± 1.0
B. longum subsp. *longum* DPC 6322	18.1 ± 1.4
B. longum subsp. *longum* DPC 6323	5.2 ± 4.8
B. longum subsp. *longum* DPC 6324	6.3 ± 0.9
B. longum subsp. *longum* 1	N.D.
B. longum subsp. *longum* 3	N.D.
B. longum subsp. *infantis* 6	N.D.
B. longum subsp. *longum* 9	N.D.
B. longum subsp. *infantis* 10	N.D.
B. longum subsp. *longum* 13	N.D.
B. longum subsp. *longum* 17	N.D.
B. longum subsp. *longum* 18	N.D.
B. longum subsp. *longum* 19	N.D.
B. longum subsp. *longum* 21	N.D.
B. longum subsp. *longum* 26	N.D.
B. longum subsp. *longum* 28	N.D.
B. longum subsp. *infantis* 31	N.D.
B. longum subsp. *longum* 36	N.D.
B. longum subsp. *longum* 40	N.D.
B. longum subsp. *longum* 43	N.D.
B. longum subsp. *longum* 45	N.D.
B. longum subsp. *longum* 47	N.D.
B. longum subsp. *longum* 49	N.D.
B. longum subsp. *longum* 52	N.D.
B. longum subsp. *longum* 53	N.D.
B. longum subsp. *longum* 56	N.D.
B. longum subsp. *longum* 58	N.D.
B. longum subsp. *longum* 59	N.D.
B. longum subsp. *longum* 61	N.D.
B. longum subsp. *longum* 74	N.D.

Legend: N.D. = Not detected.

Table 2.4 Strain, presence of *lanA* gene and presence of lantibiotic phenotype of the 38 *B. longum* strains used in this study.

Strain	Presence of *lanA* gene. 	Presence of lantibiotic phenotype.
B. longum subsp. *longum* DPC 6315	+	-
B. longum subsp. *longum* DPC 6316	-	-
B. longum subsp. *longum* DPC 6317	-	-
B. longum subsp. *longum* DPC 6318	-	-
B. longum subsp. *infantis* DPC 6319	-	-
B. longum subsp. *longum* DPC 6320	-	-
B. longum subsp. *longum* DPC 6321	-	-
B. longum subsp. *longum* DPC 6322	-	-
B. longum subsp. *longum* DPC 6323	-	-
B. longum subsp. *longum* DPC 6324	-	-
B. longum subsp. *longum* 1	-	-
B. longum subsp. *longum* 3	+	-
B. longum subsp. *infantis* 6	+	-
B. longum subsp. *longum* 9	+	-
B. longum subsp. *infantis* 10	-	-
B. longum subsp. *longum* 13	-	-
B. longum subsp. *longum* 17	-	-
B. longum subsp. *longum* 18	-	-
B. longum subsp. *longum* 19	-	-
B. longum subsp. *longum* 21	-	-
B. longum subsp. *longum* 22	-	-
B. longum subsp. *longum* 26	-	-
B. longum subsp. *longum* 28	+	-
B. longum subsp. *infantis* 31	-	-
B. longum subsp. *longum* 36	+	-
B. longum subsp. *longum* 38	-	-
B. longum subsp. *longum* 40	-	-
B. longum subsp. *longum* 43	-	-
B. longum subsp. *longum* 45	-	-
B. longum subsp. *longum* 47	-	-
B. longum subsp. *longum* 49	-	-
B. longum subsp. *longum* 52	-	-
B. longum subsp. *longum* 53	-	-
B. longum subsp. *longum* 56	-	-
B. longum subsp. *longum* 58	-	-
B. longum subsp. *longum* 59	-	-

B. longum subsp. *longum* 61	-	-
B. longum subsp. *longum* 74	-	-

Legend: Presence of *lanA* gene: + depicts *lanA* gene presence (DNA band present at ~200 bp). - depicts *lanA* gene not present (no DNA band present at ~200 bp). Presence of lantibiotic phenotype: - depicts lantibiotic phenotype not present (no inhibitory action of *B. longum* supernatants against various indicator strains tested).

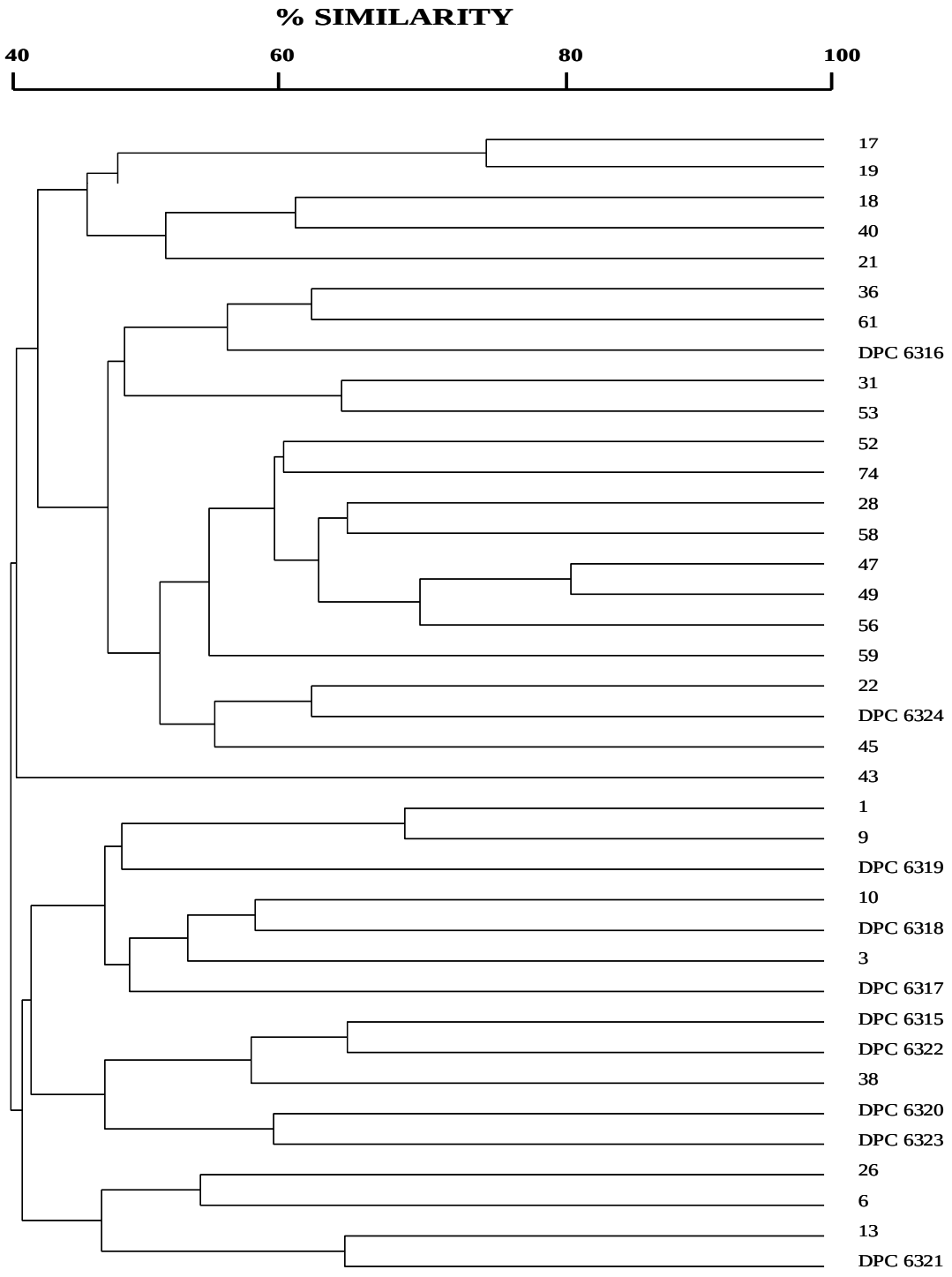


Figure 2.3 Dendogram based on computer comparisons (UPGMA clustering based on Dice correlation coefficient) of *B. longum* XbaI PFGE patterns for 38 *B. longum* strains.

CHAPTER 3

Genomic analysis of the human isolate *Bifidobacterium longum* DPC 6315.

3.1 ABSTRACT

B. longum DPC 6315 was previously found to produce an exopolysaccharide (Chapter 2), produce CLA (Barrett *et al.*, 2007) and contain a lantibiotic prepeptide *lanA* gene (Chapter 2). Based on these findings the strain was considered to have potential as a probiotic strain and as a result its genome was sequenced. The sequencing of the draft genome of *B. longum* DPC 6315 revealed it is 2.4 Mb in size with a 59% G + C content, encoding 10 scaffolds, comprised of 90 large contigs. Four predicted rRNA operons were found to be encoded within the genome. In addition, two plasmids of approximately 10.2 kb and 8.2 kb respectively were identified which were found to be almost identical (~99.5%) to previously isolated *B. longum* plasmids pNAC3 (Corneau *et al.*, 2004) and pNAL8L (Guglielmetti *et al.*, 2007). These plasmids were found to contain genes encoding replication, mobilisation and transposase functions along with a host of hypothetical proteins. The draft genome of *B. longum* DPC 6315 also contains the genes necessary for EPS, lantibiotic production and the myosin cross-reactive antigen (*mcra*) gene believed to be involved in CLA production in *Lactobacillus reuteri* PYR8 (Rosson *et al.*, 2004). *B. longum* DPC 6315 was found to produce an EPS consisting of 12:6:3:1 of mannose: glucose: galactose: rhamnose, and to display a ropy phenotype in mMRS with 7% (w/v) lactose, sucrose and glucose broth. A putative EPS operon (~16 kb) was identified in the genome of *B. longum* DPC 6315 – this potentially contained all the genes necessary for EPS biosynthesis i.e. chain length determination and export (Audy *et al.*, 2010). These included genes coding for glycosyltransferases including a pGTF gene (*wblE*) and genes *wblB and wblC* involved in the synthesis of the EPS repeating subunit,

and the genes *wblA* involved in EPS repeat unit polymerization and *wblD* involved in chain length determination.

3.2 INTRODUCTION

B. longum DPC 6315 was previously isolated from the faecal sample of a healthy 25 year old human (Barrett *et al.*, 2007), and was found in the study Barrett et al. (2007) and in Chapter 2 of this thesis to convert 60% of free linoleic acid to CLA *in vitro*. It has also been found to survive passage through the murine intestine (Wall *et al*., unpublished results) having faecal excretion rates of ~4×10^4 CFU/g. It has also been reported to possess an EPS phenotype in mMRS with 5 and with 7% (w/v) lactose broth and on agar plates (Chapter 2), and contain the pGTF *wblE* gene. It was also previously found to contain the lantibiotic prepeptide gene *lanA* (Chapter 2). For these reasons the probiotic traits of the strain were investigated in more detail using genomic analysis and characterization.

As previously described in Chapter 1, the first *B. longum* genome to be sequenced and become publicly available was the genome of *Bifidobacterium longum* NCC2705 (Schell *et al.*, 2002). At present, there are 10 published complete *B. longum* genomes (http://www.ncbi.nlm.nih.gov.library.ucc.ie/genome/genomes/183) ranging in size from 2.26 to 2.83 Mb with a G + C content spanning 59.8% to 60.3%. Of the 10 complete *B. longum* genomes, three contain two plasmids while the *B. longum* NCC2705 genome contains only a single plasmid. The draft genome of *B. longum* DPC 6315 appears to be the first *B. longum* genome described to contain genes encoding EPS, lantibiotic production and genes believed to be involved in CLA production.

EPS produced by *Bifidobacterium* species are generally heteropolysaccharides of which glucose, galactose and rhamnose are commonly present (Abbad Andaloussi *et al.*, 1995; Audy *et al.*, 2010; Salazar *et al.*, 2009). They are generally synthesized by an

intracellular pathway linked to the central carbon metabolism of the producing cell (de Vos, 1996; De Vuyst & Degeest, 1999; Ramos *et al.*, 2001). One process that is central to generating the activated sugars is the conversion of glucose-6-phosphate to glucose-1-phosphate by phosphoglucomutase (Degeest & De Vuyst, 2000). Glucose-1-phosphate reacts with UTP to generate UDP-glucose, which can be incorporated into the nascent EPS repeating unit (Sjöberg & Hahn-Hägerdal, 1989). The oligosaccharide repeating unit is first assembled by the sequential transfer of sugar residues onto a lipophilic carrier by specific glycosyltransferases. The C-terminal domain of the pGTF catalyses the addition of the first sugar 1-phosphate to a lipophilic carrier molecule for initiation of EPS repeating unit assembly - then subsequent glycosyltransferases catalyse glycosidic linkages between the oligosaccharide units.

Some genes and their predicted protein products involved in the biosynthesis of galactose 1-phosphate and glucose 1-phosphate and their conversion to EPS precursors such as UDP-glucose, UDP-galactose and dTDP-rhamnose have been located in the fully sequenced *B. longum* subsp. *longum* NCC2705 and DJO10A genomes (Audy *et al.*, 2010). All functions necessary for EPS biosynthesis were found flanking the pGTF in *B. longum* subsp. *longum* CRC 002. Overall, seven predicted gene products could be involved in heteropolysaccharide production, with putative biological functions spanning biosynthesis of the repeating unit (WblB, WblC and WblE), transport (Wzx), polymerisation (WblA) and chain length determination (WblD, Wzb) (Audy *et al.*, 2010).

In *B. longum* subsp. *longum* CRC 002, the *wblA* gene is believed to be involved in EPS repeat unit polymerization, whereas genes *wblB, wblC* and *wblE* are believed to be glycosyltransferases that could be involved in biosynthesis of the repeating unit (Audy *et*

al., 2010). The gene *wblD* is predicted to encode a protein involved in chain length determination, whereas gene *wzx*, which was located 3 kb from *wblA* in *B. longum* subsp. *longum* CRC 002 encodes a product with the topology of a 'flippase' type transporter (Audy *et al.*, 2010). This is believed to translocate the EPS-repeating sugar subunits across the membrane so that the carbohydrate moiety is on the outside of the cell. Gene *wzb* is predicted to encode the protein tyrosine phosphatase, completing all the activities essential for chain length determination (Audy *et al.*, 2010).

As previously mentioned in Chapter 2, the first bifidobacterial lantibiotic was isolated from *B. longum* DJO10A (Lee *et al.*, 2008). This lantibiotic operon was identified as a 10.2 kb gene cluster. It was also reported in that study, that this unique region was not identified in the *B. longum* NCC2705 genome. It has been suggested that selective pressure leads to the lantibiotic gene cluster being lost from the genome, as lantibiotic production would be of benefit for microbial competition in the intestine but of no value to a microbe in pure culture grown in the laboratory, as the pure culture is not competing with other cultures under these growth conditions (Lee *et al.*, 2008). Furthermore, that study revealed that a pure culture growth of *B. longum* DJO10A isolates (~1000 generations in a typical laboratory medium without pH control) that lost the regions involved in lantibiotic production format a frequency of 40%. This also rendered it sensitive to the lantibiotic produced by the original strain *B. longum* DJO10A, as the cluster that was lost also contains the immunity gene to protect the cells from the lantibiotic activity. The location of the lantibiotic region was between two IS30 elements, suggests a role for the IS elements in deletion events (Lee *et al.*, 2008).

In the study Lee et al. (2011), it was reported that lantibiotic production by *B. longum* DJO10A only occurs on agar and not in broth media which was consistent with the observation that the lantibiotic production and immunity genes, *lanADMIT*, were highly up-regulated on agar but down-regulated in broth. Quantitative real-time PCR analysis of total RNA samples from broth and agar revealed that the *lanA* gene was up-regulated more than 9 times higher on agar. This observation was also confirmed by Northern hybridization analysis substantiating that *lanA* is induced during growth on agar media.

The lantibiotic produced by *B. longum* DJO10A was collected from agar cultures and purified and it was found to have a broad spectrum of inhibition against various gram positive and some gram negative bacteria (Lee *et al.*, 2011). *B. longum* DJO10A did not show any inhibition to its own lantibiotic, which would suggest that the self-immunity function encoded by *lanI* was functional. Using this purified lantibiotic it was possible to induce *B. longum* DJO10A lantibiotic production in broth culture. Indeed, *lanA* gene expression was greatly increased in a dose-dependent fashion, confirming that increasing the external signal in broth cultures induces transcription of *lanA*.

As discussed in Chapter 1, CLA can have many beneficial effects on human health. Many different species of *Bifidobacterium* have been reported to produce CLA from linoleic acid (Barrett *et al.*, 2007; Coakley *et al.*, 2003; Rosberg-Cody *et al.*, 2004). The *mcra* gene has been suggested to be a *cis*-9, *trans*-11 CLA forming isomerase in *Lactobacillus reuteri* PYR8 (Rosson *et al.*, 2004). In *Streptococcus pyogenes* M49 the MCRA protein was reported to be an FAD-containing enzyme, which acts as a hydratase

on *cis-9* and *trans-11* double bonds of C-16, C-18 unsaturated fatty acids producing corresponding 10-hydroxy and 10, 13-dihydroxy fatty acids (Volkov *et al.*, 2010).

In this Chapter, the potential probiotic characteristics of *B. longum* DPC 6315 were examined using both genomic and phenotypic techniques to identify the genes responsible.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains, growth conditions, and media.

The bacterial strains used in this study *B. longum* DPC 6315 and *B. longum* DPC 6316, were previously isolated in the study of Barrett et al. (2007). The strains were grown on mMRS (Difco) medium supplemented with 0.05% (w/v) cysteine hydrochloride (Sigma Aldrich), 1.5% (w/v) agar (Oxoid) with 7% (w/v) lactose, sucrose, glucose, and in mMRS with 7% (w/v) lactose, sucrose and glucose broths to detect for the EPS phenotype. *B. longum* DPC 6315 was also grown in Brain heart infusion (BHI) media (Merck, Darmstadt, Germany) and the EPS produced by the strain was extracted for EPS characterisation. The agar plates and broths were incubated anaerobically (anaerobic jars with Anaerocult A gas packs (Merck) at 37°C for 72 h.

3.3.2 Nucleic acid extraction and manipulation.

Chromosomal DNA preparations were isolated from stationary phase cultures (~48 h) of *B. longum* DPC 6315 as outlined by Vincent et al. 1998 (Vincent *et al.*, 1998). DNA extraction from agarose gels and PCR purification were performed with the Qiagen gel extraction kit (Qiagen), the QIAprep spin miniprep kit (Qiagen), and the PCR purification kit (Qiagen), respectively. DNA was quantified using a Thermo Scientific nanodrop 1000 (Mason Technology, Ireland).

3.3.3 DNA sequencing.

A draft genome sequence of *B. longum* DPC 6315 was obtained by ROCHE 454 pyrosequencing using GS-FLX titanium chemistry technology. Primers were designed to

close any sequence gaps that were present in the *B. longum* DPC 6315 chromosomal and plasmid genome. The assembled draft genomes were subjected to automatic gene calling using GAMOLA (Altermann & Klaenhammer, 2003) and GLIMMER (Delcher *et al.*, 1999). Briefly, potential protein-, rRNA-, and tRNA-coding genes were predicted by GLIMMER3, RNAmmer (Lagesen *et al.*, 2007), and tRNAscan-SE, respectively (Lowe & Eddy, 1997). Artemis software (Rutherford *et al.*, 2000) was used to identify potential genes of interest in the *B. longum* DPC 6315 genome. Artemis comparison tool (ACT) was used to compare the *B. longum* DPC 6315 sequenced genome with the published *B. longum* NCC2705 genome (Schell *et al.*, 2002), to identify regions of difference between the two genomes. Putative genes in the regions of difference within the *B. longum* DPC 6315 genome were further identified by BLASTP and domain analysis.

3.3.4 Phenotypic analysis of EPS producing *B. longum* DPC 6315.

For the detection of the EPS phenotype, strains were incubated for 72 h in 30ml mMRS with 7% (w/v) lactose broth. The mMRS with 7% (w/v) lactose broth was centrifuged at 3,500 *g* × 10 min. In strains possessing an EPS positive phenotype, the cell pellet was detected at the bottom of the tube and the EPS layer suspended in the supernatant. In a strain with an EPS negative phenotype the cell pellet was detected at the bottom of the tube and the supernatant remained clear (Fig. 3.8).

3.3.5 Viscosity measurements of an EPS positive and EPS negative phenotype.

The viscosity of the cultures was determined as follows - the strains were inoculated at 2% (v/v) into 30 ml mMRS with 7% (w/v) lactose broth for 72 h at 37°C.

These broths were then centrifuged at 3,500 *g* × 10 min and the viscosities of the supernatants measured. Viscosity was measured using an AR-G2 rheometer (TA instruments, Crawley, United Kingdom) fitted with a 60-mm aluminium parallel plate. Following equilibration for 5 min at 20°C, the shear rate was increased from 0.01 to 300 s^{-1}, held for 1 min at 300 s^{-1}, and then decreased from 300 to 0.01s^{-1}. Samples were held at 20°C throughout the run.

3.3.6 Immunological analysis.

Agglutination tests were performed using *Streptococcus pneumoniae* type 37-specific antisera (Statens, Serum Institut, Denmark) as previously reported, with slight modifications (Walling *et al.*, 2005). Briefly, overnight cultures (24 h) were centrifuged (at 13,000 × *g* for 5 min), the resultant pellet resuspended in an equal volume of high-performance liquid chromatography (HPLC) water. Subsequently, 5 µl of antiserum was added and incubated for 30 min at 4°C. When agglutination occurs, cells form agglomerates observed using phase-contrast microscopy.

3.3.7 EPS isolation.

EPS was isolated from *B. longum* DPC 6315 cultures grown in mMRS with 7% (w/v) lactose broth for 72 h at 37°C following the isolation method of Mårtensson et al. (2002) with the following modifications. The pH of the samples were adjusted to pH 6.2 with 4M NaOH and then overnight hydrolysis was performed using 0.2 mg/ml proteinase K (Sigma-Aldrich, Wicklow, Ireland) at 37°C. To terminate the reaction, the mixture was heated at 90°C for 10 min and centrifuged at 4,000 × *g* for 30 min (Sorvall®LegendRT,

Thermo Scientific, Loughborough, UK). The supernatant was then collected and precipitated with 4 volumes of chilled ethanol and agitated (100 rpm) overnight at 4°C. To recover the precipitate, the mixture was centrifuged at 4,500 × *g* for 30 min. The pellet was dissolved in 10 ml of sterile deionised water and dialysed (molecular mass cut-off of 12, 000 Da) against deionised water for 3 d at 4°C (with two daily washing steps). The mixture was lypholised (VirTis AdVantage^{TM} Freeze Dryer, SP Industries, NY, USA) and the generated powder was kept at -20°C for further analysis.

3.3.8 EPS characterization.

The monosaccharide composition of isolated freeze-dried EPS was determined by sugar compositional analysis using GC-MS analysis (Price, 2004). Prior to analysis, EPS samples were refluxed in 2N trifluoroacetic acid (TFA) and converted into pre-acetylated aldonitrile acetates (PAANS). EPS structure characterisations were performed by Dr. N. Price, US, Department of Agriculture, IL, USA.

3.3.9 Phenotypic analysis of *B. longum* DPC 6315 lantibiotic production.

The method described by Lee et al. (2011) was used to isolate and purify the potential lantibiotic produced by *B. longum* DPC 6315. *B. longum* DPC 6315 was inoculated onto BLIM + Fe or mMRS media supplemented with 0.5% agar (Difco) and incubated anaerobically at 37°C for 48 h. Cultured agar media were collected and crushed in a beaker, and an equal volume of 95% methanol solution (w/v) was added. After stirring of the mixture with magnetic bars at 4°C for 24 h, the agar debris and cells were removed by centrifugation at 8, 000 × *g* at 4°C for 20 min and the supernatant filtered

using a Corning 115 ml vacuum filter system with 0.22 µm diameter filter (Corning, Lowell, MA). Methanol was then removed by concentration using a Savant SPD2010 SpeedVac concentrator at 45°C until completely dried (Thermo Scientific, Waltham, MA). The dried pellets containing the potential lantibiotic were rehydrated with 0.05 volume of molecular grade water (Sigma-Aldrich Limited, Wicklow, Ireland). Further purification and concentration was conducted by size fractionation using CentriPrep filter units with 50, 30 and 10 kDa cut-off filters in sequence (Millipore, Billerica, MA).

The bioactivity of the crude lantibiotic preparation was assayed using standard well diffusion assays (Tagg & McGiven, 1971). Indicator strains used to assess zones of inhibition included *B. longum* DJ010A, *B. breve* DPC 702258, *B. breve* DPC 6330, *Bifidobacterium longum* subsp. *infantis* ATCC 15697, and the gram positive pathogens *S. aureus, Streptococcus thermophilus* ST20, *C. difficile*, *Lactococcus lactis* subsp. *lactis* LM0230 and the gram negative pathogen *Escherichia coli* DH5a.

3.4 RESULTS.

In chapter 2, *B. longum* DPC 6315 was previously found to produce an EPS, CLA (Barrett *et al.*, 2007) and contain a lantibiotic prepeptide *lanA* gene (Chapter 2). The strain was considered to have potential as a probiotic strain based on these findings and as a result its genome was sequenced.

3.4.1 DNA sequencing.

Initial automatic assembly of the 454 reads generated a non-redundant sequence of 2.4 Mb with a 59% G + C content, encoding 10 scaffolds, comprised of 90 large contigs. Four predicted rRNA operons were found to be encoded within the genome. A comparison with the published completely sequenced genome of *B. longum* NCC2705 revealed a number of significant regions of difference (Fig. 3.1). These regions of difference were then further investigated for novel genes in the *B. longum* DPC 6315 genome which revealed plasmid DNA and putative genes encoding EPS, bacteriocin and CLA production. Two plasmids of ~10.2 kb and ~8.2 kb respectively, were identified in the *B. longum* DPC 6315 genome with high identities (~99.5%) to previously isolated *B. longum* plasmids pNAC3 (Corneau *et al.*, 2004) and pNAL8L (Guglielmetti *et al.*, 2007), (Fig. 3.2). These plasmids were found to contain genes coding for replication, mobilisation and transposase functions, in addition to coding for a number of hypothetical proteins.

A putative EPS operon (~16 kb) (Fig. 3.3) was also identified in the genome of *B. longum* DPC 6315 – this potentially contained all the genes necessary for EPS biosynthesis i.e. chain length determination and export (Audy *et al.*, 2010). These genes

had high sequence identities to EPS production encoding genes located in *B. longum* CRC 002, with genes *wblE* and *wblD* having ~95% sequence identity to genes *wblE* and *wblD* found in *B. longum* CRC 002, and genes *wblA*, *wblB* and *wblC* having ~76% sequence identity to genes *wblA*, *wblB* and *wblC* found in *B. longum* CRC 002. Interestingly, the EPS operon located in the genome of *B. longum* DPC 6315 has a different gene organization compared to the EPS operon found in *B. longum* CRC 002.

A putative lantibiotic operon (~10.2 kb) was identified in the draft genome of *B. longum* DPC 6315 with ~99% sequence identity to the lantibiotic gene cluster found in *B. longum* DJO10A (Lee *et al.*, 2008). This lantibiotic gene operon was found to encode eight genes (Fig. 3.4). Lee et al. (2011) reported that this gene cluster contained all the potential genes required for lantibiotic production, as well as a dedicated two component system (*lanR2* and *lanK*), which is characteristic of lantibiotic gene clusters in other bacteria (Klaenhammer, 1993). A second predicted transcription regulator (*lanR1*) was found which is an analogous organization to the mersacidin gene cluster from a *Bacillus* species (Altena *et al.*, 2000). The potential lantibiotic produced by *B. longum* DPC 6315 was isolated and purified using a method described by Lee et al. (2011). However, no lantibiotic activity was observed from *B. longum* DPC 6315 supernatants using well diffusion assays against other *B. longum* (Chapter 2) and various gram-positive and gram-negative pathogenic indicator strains.

With regards to CLA production, an open reading frame (ORF) homologous to the MCRA protein ORF found in *B. longum* NCC2705 (Fig. 3.5) was also identified in the draft genome of *B. longum* DPC 6315. An intact DNA polymerase III protein ORF was located upstream of the MCRA ORF. The MCRA and the DNA polymerase III

proteins share ~97% and ~96% amino acid identities respectively, to the MCRA and the DNA polymerase III proteins located in the CLA producing *B. breve* DPC 6330. Interestingly, a gene encoding a hypothetical protein was located between the *mcra* and the DNA polymerase III encoding genes, that possessed ~99% amino acid identity to a narrowly conserved hypothetical protein found in *Bifidobacterium longum* subsp. *infantis* ATCC 15697 (Fig. 3.5). Located just downstream of the MCRA ORF were potential genes encoding histone acetyltransferase HPA2 protein and related acetyltransferase proteins. Further downstream was an ORF encoding a ribosomal protein with ~100% homology to the 50S ribosomal protein L1 found in *Bifidobacterium longum* subsp. *longum* JDM 301 (Fig. 3.5).

3.4.2 EPS characterization.

The EPS produced by *B. longum* DPC 6315 is most likely not of the beta glucan type EPS as the producing cells did not aggregate after treatment with *Streptococcus pneumoniae* type 37-specific antisera (Fig. 3.6 (a)) compared to the beta glucan producing *Lactobacillus paracasei* subsp. *paracasei* 338 control (Fig. 3.6 (b)).

Characterisation of the monosaccharide structure of the EPS produced by *B. longum* DPC showed it is composed of 12:6:3:1 of mannose: glucose: galactose: rhamnose whereas the EPS produced by *B. longum* CRC 002 is composed of 2:3 galactose: glucose. *B. longum* DPC 6315 displayed a ropy phenotype in mMRS with 7% (w/v) lactose, sucrose or glucose broths and agar plates. The strain was found to be most ropy when grown on mMRS with 7% (w/v) lactose. Fig. 3.7 displays the EPS phenotype of *B. longum* DPC 6315 in mMRS with 7% (w/v) lactose broth.

A clear phenotypic difference in EPS production was observed when 72 h cultures grown in mMRS with 7% (w/v) lactose broth of both *B. longum* DPC 6315 (EPS +ve) and *B. longum* DPC 6316 (EPS -ve) (Chapter 2) were centrifuged (3,500 *g* × 10 min) (Fig. 3.8). *B. longum* DPC 6315 displayed a positive EPS phenotype, giving a cloudy supernatant with the cell pellet settling at the bottom of the tube (Fig. 3.8 (EPS +ve)). In constrast, *B. longum* DPC 6316 displayed a negative EPS phenotype and yielded a clear supernatant with the cell pellet settling at the bottom of the tube after centrifugation (Fig. 3.8 (EPS –ve)). These images clearly show that in an EPS positive phenotype the EPS layer remains suspended throughout the supernatant after centrifugation (Fig. 3.8). The generated supernatants also showed differing viscosities with that from *B. longum* DPC 6315, having a significantly (P<0.001) higher value of 2.9 mPa.S compared to of 1.74 mPa.S for the control strain (Fig. 3.9).

3.5 DISCUSSION

Of the published *B. longum* genome sequences available, the genome of *B. longum* DPC 6315 appears to be the only genome that contains the genes for EPS, bacteriocin and CLA production.

The EPS produced by *B. longum* DPC 6315 appears to be a unique bifidobacterial EPS containing the monosaccharides mannose, glucose, galactose, and rhamnose in an approximate ratio of 12:6:3:1. The high levels of mannose in bifidobacterial EPS have only been previously reported for three other *Bifidobacterium* species - namely *B. longum* DSM 21062, CHCC8773 and CHCC8818 (Kildsgaard *et al.*, 2011). The EPS produced by *B. longum* DSM 21062 was found to contain the monosaccharides glucose, mannose, galactose, fucose in an approximate ratio of 107:34:1.5:1 with only a trace of rhamnose, while the EPS of *B. longum* CHCC8773 contained mannose at the highest concentration of any monosaccharide present with an approximate ratio of mannose: glucose: galactose: fucose of 23:11:3:1. The EPS produced by *B. longum* CHCC8818 also contained mannose as the monosaccharide with the highest concentration with an approximate ratio of mannose: glucose: galactose: fucose of 36:17:3:1. It is tempting to suggest that the high concentration of mannose in the EPS produced by *B. longum* DSM 21062 could possibly be responsible for the strain's immunodulatory effects. This strain was reported to possess anti-inflammatory effects in a trinitrobenzene sulfonate (TNBS)-induced colitis model in mice (Kildsgaard *et al.*, 2011). In this murine model, it reduced the TNBS-induced injury by 44% (P=0.007) when fed to mice at 1×10^9 live bacteria daily for 5 consecutive days - compared to the two other *B. longum* strains tested which were CHCC8773 and CHCC8818. It has also been previously reported that complex polymers

containing mannose (mannans) possess significant biological activity when administered to mammals particularly including the activation of the immune system following the binding of mannose to recognition molecules such as the mannose receptor (CD206) expressed on macrophages and dendritic cells (Carroll & Prodeus, 1998; Diebold *et al.*, 2002; Stahl & Ezekowitz, 1998). Likewise, it is also possible that the EPS produced by *B. longum* DPC 6315 could have positive immunodulatory effects *in vivo* as it produces an EPS with high levels of mannose.

EPS produced by some strains of LAB can be used to increase the viscosity of fermented food products (Cerning, 1995; De Vuyst, 1999; Goh *et al.*, 2005). In mMRS containing 7% (w/v) lactose broth, the EPS produced by *B. longum* DPC 6315 had a significant effect on the viscosity of the crude supernatant. These results demonstrate that under the proper conditions for EPS production and maintenance, the EPS produced by *B. longum* DPC 6315 could enhance the viscosity of a food product leading to a more desirable texture which could be of commercial value. Indeed, the EPS produced by *B. longum* DPC 6315 could possibly be used as a natural additive in food products, which are preferred by some consumers over stabilisers of plant or animal origin.

The lantibiotic gene operon identified in the genome of *B. longum* DPC 6315 was previously identified in *B. longum* DJO10A (Lee *et al.*, 2008). Using well diffusion assays and spot plate overlays, however, no antimicrobial activity of the *B. longum* DPC 6315 supernatants was found against other *B. longum* strains (Chapter 2) – nor were various Gram-positive and Gram-negative pathogenic bacteria inhibited or killed. This could be due to one of the genes essential for lantibiotic production being switched off in *B. longum* DPC 6315 or that the level of the lantibiotic produced by the strain were too

low to detect any activity against indicator strains *in vitro* – in other words being below the minimum inhibitory concentration. It is possible production of a lantibiotic *in vivo* could confer an advantage on *B. longum* DPC 6315 over other bacterial strains *in vivo*. In this respect, production of the lantibiotic might be very different in the gut compared to that seen in pure culture as the environments are so different.

The identification of the MCRA protein in the draft genome of *B. longum* DPC 6315 suggests it performs a function in the bacterial strain's conversion of linoleic acid to CLA. The MCRA and the DNA polymerase III proteins in *B. longum* DPC 6315 were found to have ~97% and ~96% identity respectively to those in *B. breve* DPC 6330, which would suggest that these proteins are highly conserved in different CLA producing *Bifidobacterium* species.

In conclusion, the identified probiotic traits in the draft genome of *B. longum* DPC 6315 make it an attractive potential probiotic bacterium strain. As it was originally isolated from adult faeces (Barrett *et al.*, 2007), and has been found to survive passage through the murine intestine (Wall *et al*., unpublished results), it has the ability to survive passage through the gastrointestinal tract which is a prerequisite for any probiotic bacterial strain. The production of EPS, CLA and the potential production of a lantibiotic by *B. longum* DPC 6315 make it a possible future candidate strain as a probiotic.

3.6 ACKNOWLEDGEMENT

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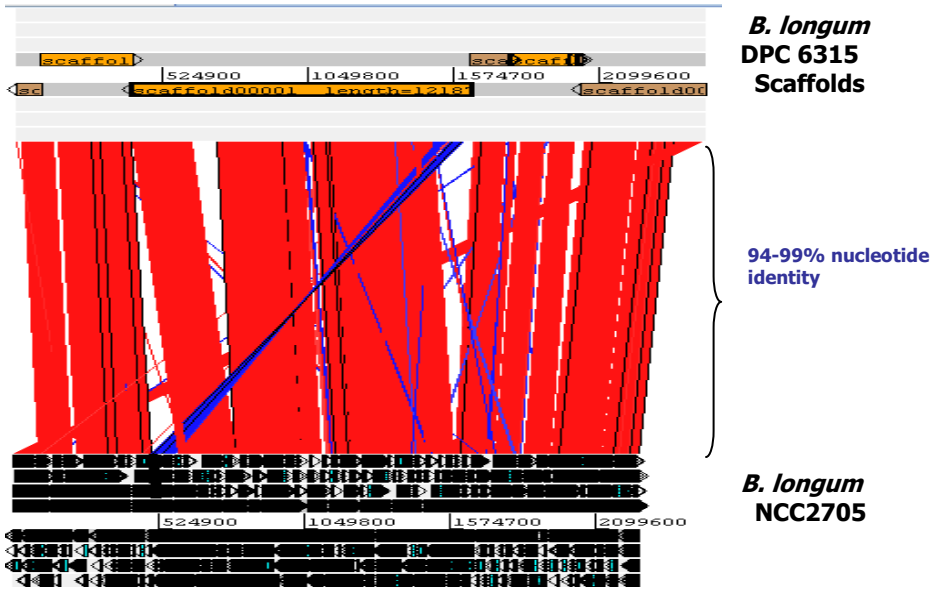


Alignment of ordered scaffolds of *B. longum* DPC 6315

and a sequenced reference genome *B. longum* NCC2705

Figure 3.1 ACT alignment of draft genome of *B. longum* DPC 6315 and *B. longum* NCC2705 (Regions of difference in white). Red lines indicate regions of similar sequence with the same orientation in both genomes. Blue lines indicate regions of similarity that have an inverted configuration.

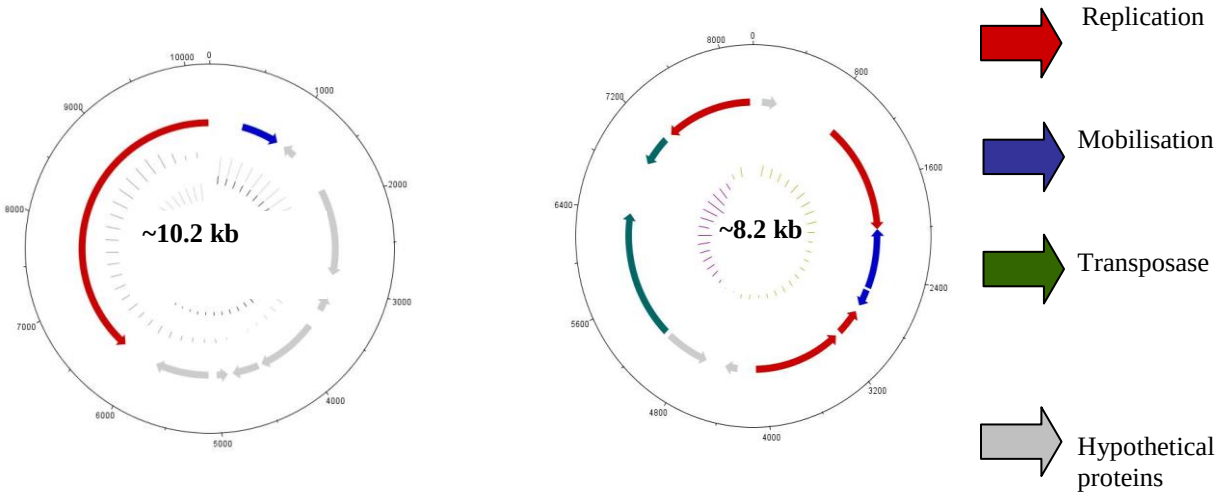
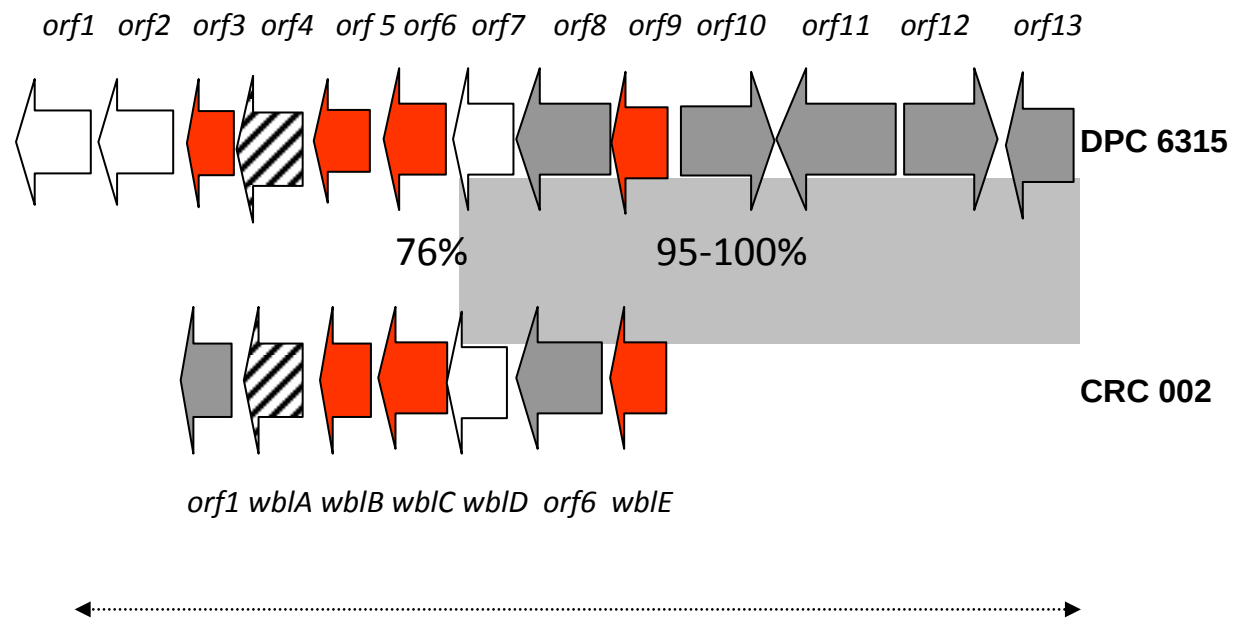


Figure 3.2 Plasmids identified in draft genome of *B. longum* DPC 6315.

Almost identical (~99.5%) to previously isolated *B. longum* plasmids pNAC3 (~10.2 kb) (Corneau *et al.*, 2004) and pNAL8L (~8.2 kb) (Guglielmetti *et al.*, 2007).



Legend:

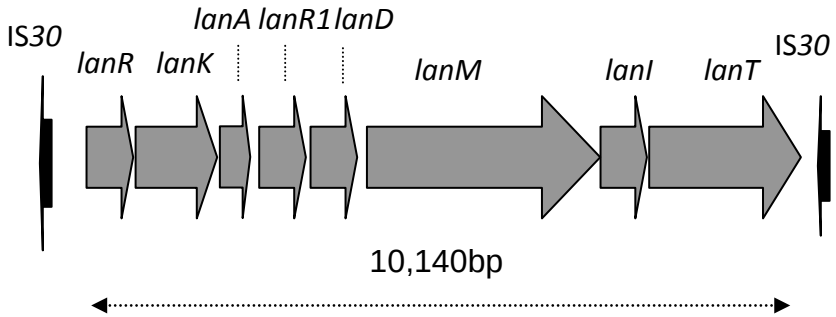
Glycosyltransferase

Polymerisation

Chain length determination

Unknown function in EPS production.

Figure 3.3 Organisation of putative EPS producing operon in *B. longum* DPC 6315. EPS operon is compared to the operon in *B. longum* CRC 002 (Audy *et al.*, 2010). Percentages are representative of nucleotide identification.



Legend:

Grey-coloured arrows indicate the component genes for:
lanA = Lantibiotic production gene.

lanD/M = Lantibiotic modifying enzyme.

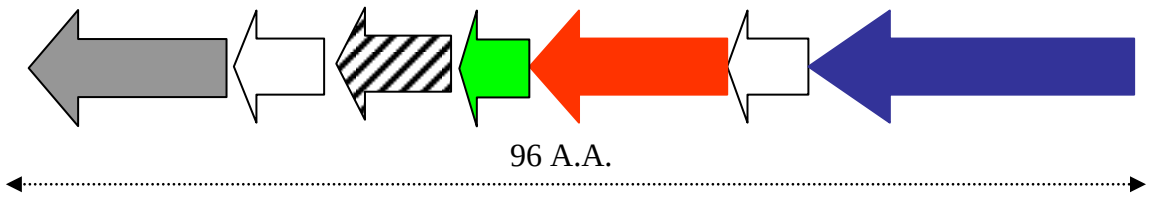
lanI = Lantibiotic immunity gene.

lanK/R = Lantibiotic response regulator.

lanT = Lantibiotic transporter containing removal activity of leader peptide.

IS = Insertion sequence

Figure 3.4 Organisation of putative lantibiotic operon in *B. longum* DPC 6315.



Legend:

Myosin cross-reactive antigen

Ribosomal protein

Hypothetical protein

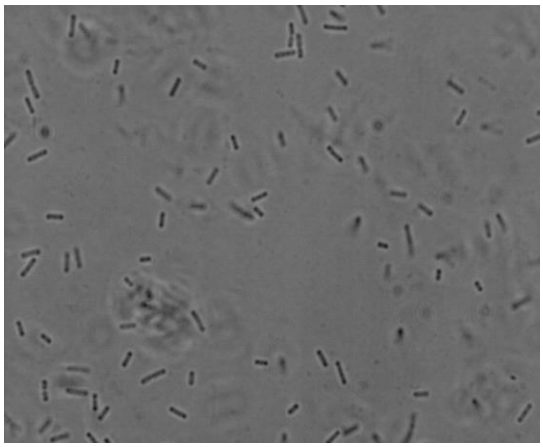
Unknown protein

DNA polymerase III

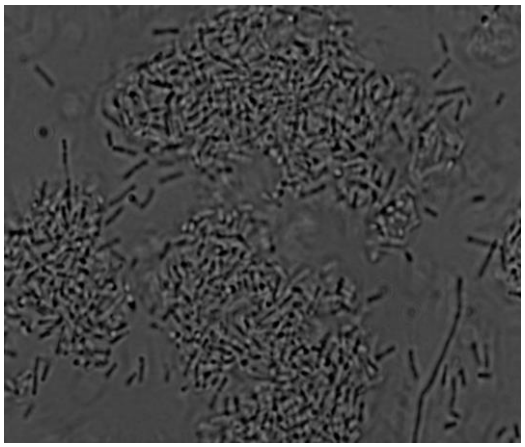
Histone acetyltransferase HPA2 & related acetyltransferases.

A.A. = Amino acid

Figure 3.5 MCRA protein and related proteins identified in the draft genome of *B. longum* DPC 6315.



3.6 (a). EPS producing *B. longum* DPC 6315 + *S. pneumoniae* type 37 antisera



3.6 (b). Beta glucan producing *L. paracasei* NFBC 338 + *S. pneumoniae* type 37 antisera

Figure 3.6 Immunological analysis of EPS producing *B. longum* DPC 6315 cells (a) compared to *L. paracasei* NFBC 338 beta glucan producing cells (b).

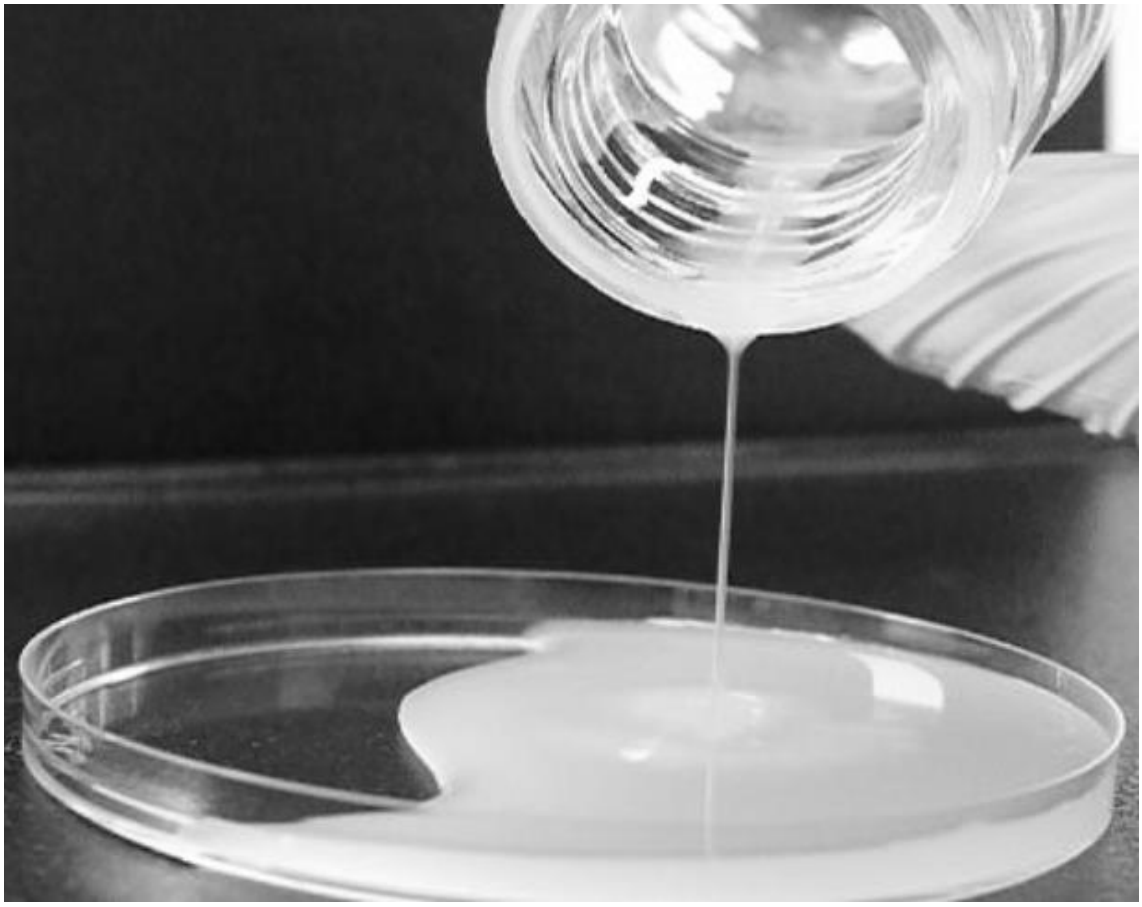


Figure 3.7 Image of *B. longum* DPC 6315 EPS phenotype in mMRS with 7% (w/v) lactose broth.

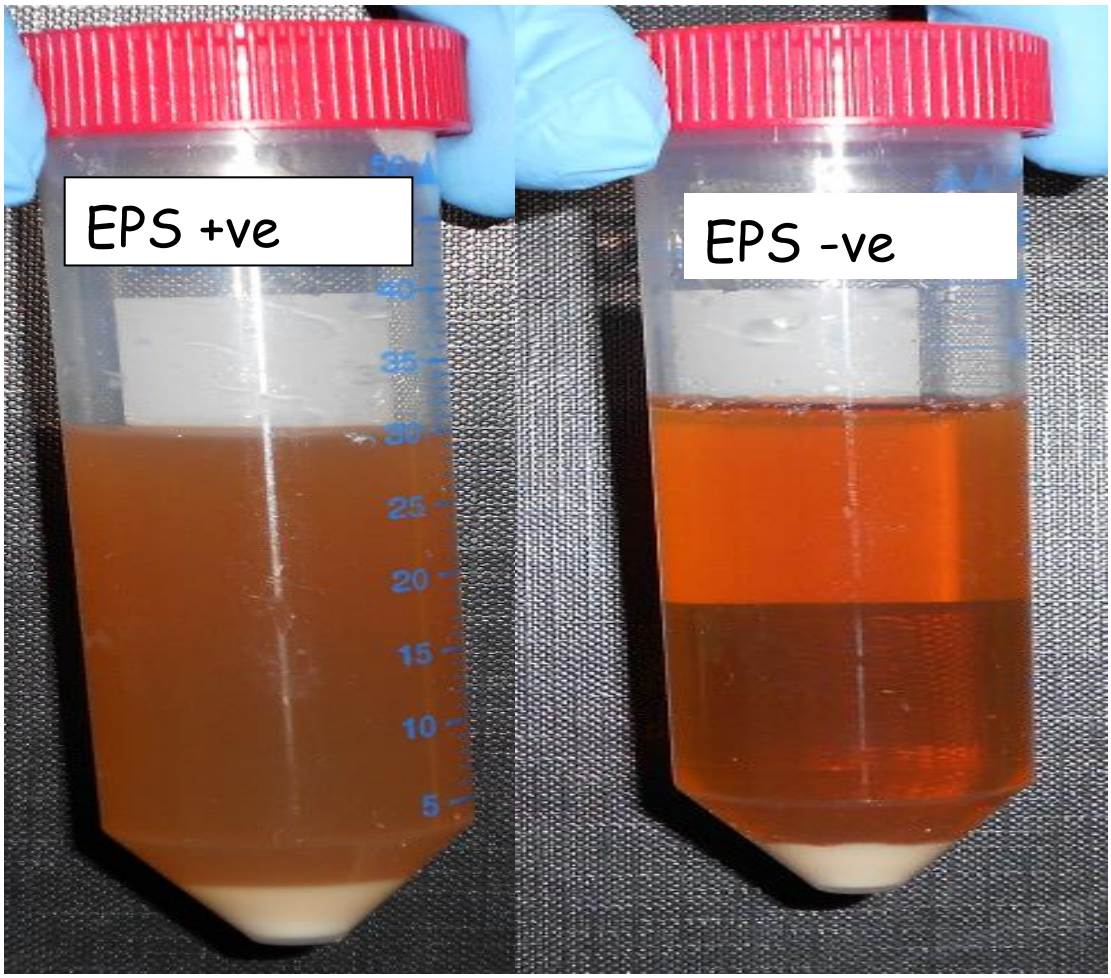
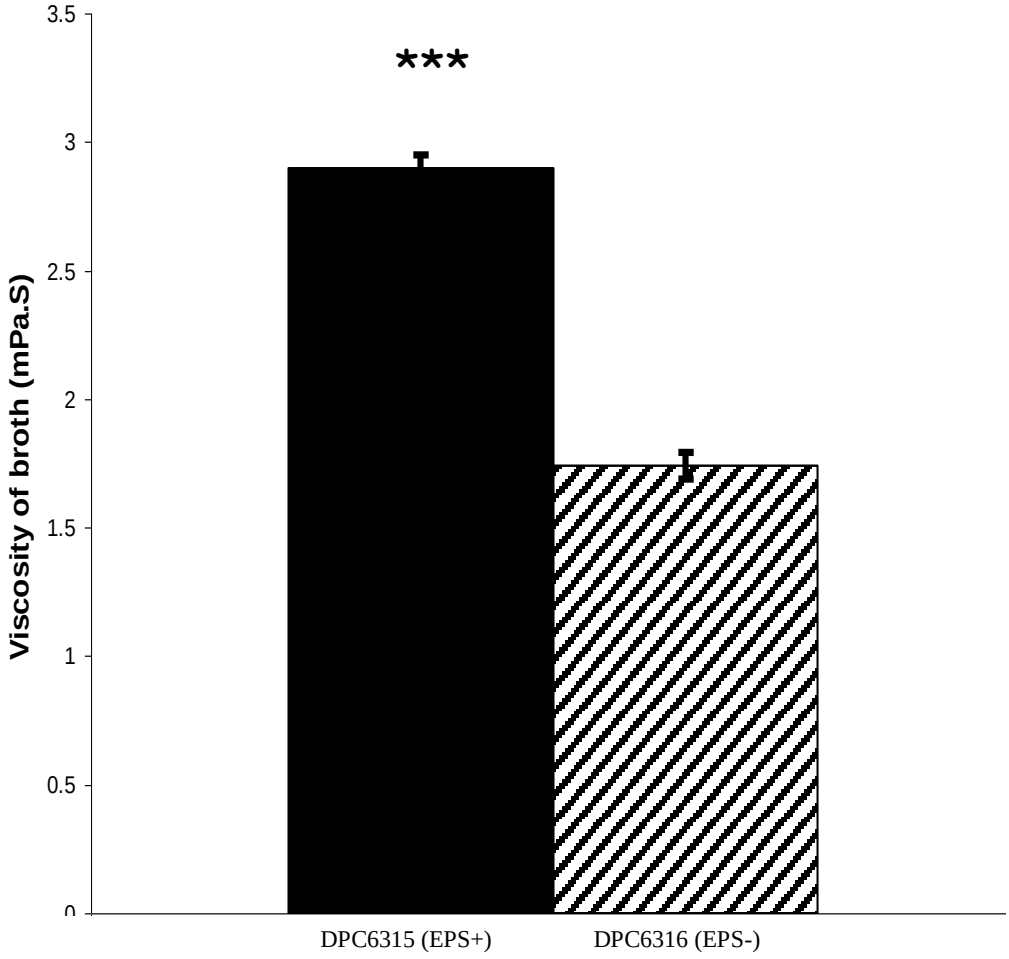


Figure 3.8 Image of *B. longum* DPC 6315 EPS positive (EPS +ve) phenotype and *B. longum* DPC 6316 EPS negative phenotype (EPS –ve).



*** ***P*<0.001**

Figure 3.9 Viscosity analysis of centrifuged crude supernatant (3,500 *g* × 10 min) EPS positive *B. longum* DPC 6315 (EPS+) and EPS negative (EPS-) *B. longum* DPC 6316 grown in mMRS with 7% (w/v) lactose broth.

CHAPTER 4

The effect of exopolysaccharide producing *Bifidobacterium longum* DPC 6315 on the functional properties of yoghurt.

4.1 ABSTRACT

The influence of the EPS-producing adjunct strain, *Bifidobacterium longum* DPC 6315 on the functional properties of yoghurt was examined. *In situ* production of EPS by *B. longum* DPC 6315 during product manufacture and up to 14 days of cold storage at 4°C was found to significantly (P<0.05) increase (from 36.9 (± 2.9) mg/l on day 0 to an average of 51.4 (± 9.4) mg/l EPS on day 7). In contrast, the concentration of EPS in the EPS containing yoghurt slightly declined on day 14, and significantly decreased on 21 (P<0.01) and 28 (P<0.01) of cold storage, to an average of 19.3 (± 4.4) mg/l EPS on day 28. The performance and viability of the yoghurt cultures were not affected by addition of *B. longum* DPC 6315, with *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* detected at 1.7×10^7 CFU/g and 2.8×10^5 CFU/g, respectively, following 28 days of storage at 4°C. The viability of the EPS-producing *B. longum* DPC 6315 was 2×10^6 CFU/g after 28 days of storage at 4°C, which is above the minimum of 10^6 CFU/g recommended by the FAO/WHO guidelines (FAO/WHO, 2002) for a probiotic bacterium in a fermented food. During yoghurt manufacture and at 14 days of storage at 4°C, a significant decrease (P<0.05) in syneresis was obtained in yoghurt containing the EPS producing strain compared with the control. No significant difference was found in viscosity after fermentation and during 28 days of storage at 4°C between the yoghurt manufactured with the EPS-producing *B. longum* DPC 6315 and the control yoghurt. Confocal laser scanning microscopy (CLSM) of unstirred yoghurt samples demonstrated that the EPS was present around the edges of the pores of the yoghurt network but not in the control yoghurt. This study demonstrates that the use of *B. longum* DPC 6315 as an adjunct culture during yoghurt manufacture resulted in lower

levels of syneresis and higher levels of EPS, and as such use of the adjunct offers some technological improvements to the product over and above its probiotic characteristics.

4.2 INTRODUCTION

Yoghurt has been consumed in many cultures as part of the daily diet for centuries. Yoghurt is a protein coagulum of a casein network in which serum, fat globules, and bacterial cells are entrapped. The distribution of the 'filling compounds' such as EPS and their interactions with casein micelles can have a positive influence on the rheological properties of yoghurt since it can strengthen the relatively weak structure of the casein network (Hassan *et al*., 2002). The addition of stabilisers of plant or animal origin to natural yoghurts is prohibited in most European Union countries (Degeest & De Vuyst, 1999), and consequently EPS produced by LAB have been commonly used to enhance the texture and viscosity of dairy products (Costa *et al*., 2012).

The function of EPS in fermented milk and yoghurt is affected by a number of factors including monosaccharide composition, charge, linkage types, branching, molecular weight, and the ability to interact with milk protein (Duboc & Mollet, 2001; Kleerebezem *et al*., 1999; Ruas-Madiedo *et al*., 2002a). EPS produced by LAB such as lactobacilli, streptococci and lactococci can be used as a natural thickener, to increase the viscosity and to decrease syneresis of fermented milk products (Cerning, 1995; De Vuyst, 1999; Goh *et al*., 2005). Syneresis or whey separation is the spontaneous appearance of whey on a milk gel surface and is considered a major defect in the yoghurt industry (Tamime & Robinson, 2007b). Studies carried out by London et al. (submitted 2013) and Kearney et al. (2011) found that EPS produced by different *Lactobacillus* strains used in yoghurt fermentations increased the viscosity and decreased the syneresis in the product.

Bifidobacteria in general have been reported to exhibit poor growth in dairy based foods when compared with traditional LAB used in fermented dairy products, which

could possibly hinder their potential applications (Prasanna *et al*., 2011). In this respect, they generally require long fermentation times, anaerobic conditions, and low redox potential for growth when grown in milk (Gomes & Malcata, 1999; Hughes & Hoover, 1995; Jayamanne & Adams, 2009). In addition, Samona and Robinson (1994), reported that the growth of *Bifidobacterium* species was adversely affected by the presence of yoghurt cultures. In contrast, the yoghurt starter culture *S. thermophilus* was reported to stimulate the growth of *Bifidobacterium* species in yoghurt as it acts as an oxygen scavenger by reducing the level of dissolved oxygen in the yoghurt (Ishibashi & Shimamura, 1993). *L. delbrueckii* subsp. *bulgaricus* has also been found to have a stimulatory effect on *B. bifidum* possibly due to its proteolytic activity (Rybka, 1994). A further major drawback to the use of bifidobacteria as probiotic cultures in the production of yoghurt and fermented milk is their poor survival at low pH (the pH of yoghurt ranges from 4.2 to 4.7) (Dave & Shah, 1998; Hughes & Hoover, 1995; Lamoureux *et al*., 2002; Roy *et al*., 1997). The addition of bifidobacteria to yoghurt can also improve its health enhancing value (Kailaspathy & Rybka, 1997). For example, yoghurt containing *Bifidobacterium* species have been reported to improve lactose utilization (He *et al*., 2007) and increase HDL cholesterol or (the so called 'good cholesterol') following consumption (Kiessling *et al*., 2002). Wang et al. (2004) also reported that the consumption of yoghurt for six weeks containing *B. lactis* Bb12 and *Lactobacillus acidophilus* La5 by patients infected with *H. pylori* significantly decreased the values of the urea breath test which is the standard test for measuring *H. pylori* infection. This study also reported a significant decrease in gastritis activity and *H. pylori* from endoscopic biopsies of gastric mucosa taken from the antrum of 14 patients who had

consumed the *B. lactis* Bb12 and *L. acidophilus* La5 containing yoghurt (Wang *et al*., 2004). In another study, Matsumoto and Benno (2004) reported that the consumption of yoghurt containing *Bifidobacterium lactis* LKM512 for two weeks reduced gut mutagenicity by increasing the levels of polyamines in healthy adult subjects. Colombel et al. (1987) reported that consumption of yoghurt containing *B. longum* decreased faecal excretion of clostridia and abolished erythromycin-induced abdominal discomfort. Regular consumption of yoghurt (400-500g/week) containing at least 1×10^6 CFU/g of *Bifidobacterium* species which are capable of surviving in the upper GIT, is recommended to achieve therapeutic benefits (Tamime *et al*., 1995).

In this study the effects of the adjunct strain *B. longum* DPC 6315 were examined during yoghurt manufacture. This strain was selected based on its ability to produce EPS and give a ropy phenotype. In addition, the strain has additional potential as a probiotic strain in that it produces CLA from linoleic acid and also has the potential to produce a lantibiotic based on its genotype.

4.3 MATERIALS AND METHODS

4.3.1 Starter microorganisms

B. longum DPC 6315 was isolated from the human GIT (Barrett *et al*., 2007) and identified as an EPS producer (Chapter 2). In this study, *B. longum* DPC 6315 was used as a ropy and EPS-producing adjunct culture during yoghurt manufacture. Prior to yoghurt manufacture, *B. longum* DPC 6315 was propagated three times in *Lactobacillus* MRS (Difco) containing 0.05% (w/v) L-cysteine hydrochloride (Sigma) at 37°C, anaerobically. The thermophilic yoghurt starter, CH-1 (non-EPS-producing) consisted of a defined mixed single strain of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in a freeze-dried pellet form (Chr. Hansen, Horsholm, Denmark). Before use, the CH-1 culture was activated by adding a 50 U sachet (consisting of ~ 1×10^6 CFU/g of *S. thermophilus* and ~ 1×10^8 CFU/g of *L. delbrueckii* ssp. *bulgaricus*) to 500 ml of sterile 14% (w/v) reconstituted medium-heat skimmed milk (RSM; Kerry Ingredients Ltd., Kerry, Ireland) and agitated for 15 min to achieve a homogenous culture, according to the manufacturer's instructions. The freeze-dried cultures were stored at -20°C until use.

4.3.2 Yoghurt Manufacture

The fermentation substrate consisted of 14% (w/v) RSM (Kerry Ingredients Ltd.) supplemented with 1% (w/v) yeast extract. The fermentation substrate was heat treated at 121°C for 5 min prior to inoculation. The mixture was then cooled to 37°C and 3 l aliquots were transferred aseptically into separate sterile bottles. Overnight cultures (18 h) of *B. longum* DPC 6315 were centrifuged (Sorvall® RC-5B Plus, Thermo Scientific, Waltham, MA, USA) at 10,000 × *g* for 10 min at 4°C, washed with sterile Milli-Q water

and resuspended with an aliquot of the fermentation substrate. Into separate aliquots of RSM, representing the control and *B. longum* DPC 6315 containing yoghurts, fermentation substrate was inoculated at 0.2% (v/v) with either the activated CH-1 culture (control) or the activated CH-1 culture in combination with *B. longum* DPC 6315 and agitated for 10 min to achieve adequate mixing. The samples were distributed in 125 ml sterile plastic containers (VWR International, Dublin 15, Ireland) and incubated in an anaerobic gas chamber (Don Whitley Anaerobic Cabinet MACS 500, Davidson and Hardy, Dublin 4, Ireland) at 37°C. The fermentation was performed under anaerobic conditions (as oxygen negatively affects the growth of *B. longum* DPC 6315, as it is an anaerobic culture (Barrett *et al*., 2007)) for 6.5 h and was terminated at pH 4.7 when the yoghurt samples were immediately stored at 4°C for 28 days. All data are based on triplicate yoghurt trials.

4.3.3 Determination of culture viability

For enumeration of viable microorganisms, aliquots of milk or yoghurt, taken at selected time points, were serially diluted in maximum recovery diluent (MRD; Oxoid c/o Fannin Healthcare, Dublin, Ireland) and plated onto the appropriate selective medium for each strain. *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* were enumerated on LM17 and MRS (adjusted to pH 5.2; Difco), containing 2% (w/v) agar (Oxoid), respectively (Kearney *et al*., 2009). *B. longum* DPC 6315 was enumerated anaerobically on mMRS (Difco) medium supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma), 1.5% (w/v) agar (Oxoid) containing mupirocin (50 mg/l) (Oxoid) from

antimicrobial susceptibility test discs as previously described by (Rada, 2000) at 37°C for 48 h.

4.3.4 Isolation and quantification of EPS

The isolation and quantification of EPS in yoghurt was performed as previously reported (Amatayakul *et al*., 2006; Zisu & Shah, 2003). A 25 ml sample of yoghurt was diluted (1:1) with Milli-Q water. The casein fraction was precipitated by adding 4 ml 0.2 M trichloroacetic acid (TCA; Sigma Aldrich), followed by centrifugation (Sorvall® Legend RT, Thermo Scientific) at 3,310 × *g* for 30 min at 4°C to remove the precipitate and the bacterial cells. The pH of the supernatant was adjusted to 6.8 with 4 M NaOH, boiled in a sealed container for 30 min and centrifuged (3,310 × *g* for 30 min at 4°C) to remove the whey proteins. An equal volume of chilled ethanol was then added to the supernatant to precipitate the carbohydrates and agitated (100 rpm) overnight at 4°C. The overnight sample was centrifuged at 3,310 × *g* for 30 min at 4°C - the carbohydrate pellet was then resuspended in 10 mL of Milli-Q water and sonicated for 1 h at room temperature using a sonication bath (Decon FS100B, Decon Laboratories, East Sussex, UK). The sample was vortex mixed to a uniform solution and dialysed (molecular mass cut-off of 1200 Da) against deionised water at 4°C for 7 days (with two daily changes of water). The dialysate was stored at -20°C until assayed. The EPS concentration in the suspension was quantified using the phenol-sulphuric method and was expressed as the glucose equivalent (DuBois *et al*., 1956). The concentrations of EPS for *B. longum* DPC 6315 was determined by subtracting the total amount of glucose detected in unfermented culture medium (which was used as a blank) from total amount of glucose detected in the

inoculated fermentation medium. The concentration of EPS in the yoghurt samples was determined by subtracting the total amount of glucose detected in the control yoghurt (used as a blank) from the total amount of glucose detected in the *B. longum* DPC 6315 containing yoghurt, at each storage point assayed.

4.3.5 Chemical and physical properties of yoghurt

The pH change of yoghurt during fermentation and storage was monitored using a pH meter (model MP220, Mettler-Toledo, Greifensee, Switzerland), with a calibrated electrode (Mettler-Toledo InLab® 413, Mettler-Toledo). The titrable acidity of the yoghurt was assessed by adding one drop of phenolphthalein (Sigma Aldrich) to 10 g of yoghurt and titrating with 0.1 M NaOH (Sigma Aldrich) until a light rose to pink colour persisted (Tamime & Robinson, 2007a). Titratable acidity was expressed as percentage of lactic acid, calculated as follows:

$$\% \text{ Lactic acid} = \text{Alkali (ml)}/10 \quad (1)$$

The extent of syneresis during storage was assessed by a centrifugation method outlined in a previous study (Amatayakul *et al*., 2006). Synereis was expressed as:

$$\text{Syneresis (\%)} = \frac{\text{Weight of the whey separated from the gel}}{\text{Initial weight of the yoghurt sample}} \times 100 \quad (2)$$

Viscosity measurements of the yoghurt samples were perofrmed using an AR-G2 ex rheometer (TA Instruments, Crawley, UK) equipped with a parallel plate geometry (diameter 60 mm). Prior to analysis, the samples were stirred with a spatula 20 times to achieve a homogenous mixture. The samples were then pre-sheared at 200 s^{-1} for 1 min to remove any residual structure (Vasiljevic *et al*., 2007), allowed to equilibrate for 1 min, sheared from 0.01 to 200 s^{-1} over 2 min, held at 200 s^{-1} for 1 min and sheared from 200 to 0.01 s^{-1} over 2 min. All tests were performed at 20°C.

4.3.6 Confocal laser scanning microscopy (CLSM).

The microstructure of the yoghurt samples was examined following 28 days of storage following staining using CLSM. The labelling fluorescent stain, Fast Green FCF (Sigma Aldrich), was used to label the protein. A 20µl volume of a 0.1 g/l aqueous solution of Fast Green FCF was applied to the surface of the yoghurt sample. Fast Green FCF was applied to the surface of the yoghurt sample. Fast Green FCF labelled the protein and displayed the protein as a red colour. Fast Green FCF label proteins when excited at 633 nm (Auty *et al*., 2001). The lectin Wheat Germ Agglutinin Alexafluor 555 conjugate (WGA; Invitrogen) – was used to visualise the bacterial EPS. The Wheat Germ Agglutinin 555 stock solution was freshly prepared by dissolving 1 mg of the lectin Wheat Germ Agglutinin Alexafluor 555 conjugate in a mixture of 1.5 ml phosphate buffer at pH 6.3 and 0.5 ml ethanol. The lectin Wheat Germ Agglutinin Alexafluor 555 labels EPS when excited at 561 nm. The EPS was labelled with Wheat Germ Agglutinin Alexafluor 555 and displayed the EPS as a blue colour. To label the bacteria, the SYTO9 solution was used (excitation and emission maxima, 480 and 500 nm respectively) and

penetrated both viable and non-viable bacteria. A 10 µl aliquot of the SYTO9 solution was added to the surface of the yoghurt. The argon ion laser was used to generate the 488 line for excitation and visualization of the bacteria. Syto 9 labelled live and dead bacteria and the cultures were visualized as a green colour. The argon ion laser was used to generate the 488 line for excitation and visualisation of the bacteria. All samples were gently stirred after adding the dyes. Imaging was performed using a Leica TCS SP3 confocal laser scanning microscope (Leica Microsystems, Heidelberg GmbH, Mannheim, Germany) with a 63x oil immersion objective (A_N = 1.4). RGB colour images (24 bits), 512 × 512 pixel resolution, in TIFF format were acquired. A minimum of 4 z-stacks were taken per sample with representative cross sections of micrographs shown.

4.3.7 Statistical analysis

Data from triplicate trials were subjected to unpaired T-test analysis. Results are considered significant as follows:

* P<0.05; ** P <0.01; *** P<0.001.

4.4 RESULTS

4.4.1 Culture viability during yoghurt fermentation and storage

Prior to yoghurt fermentation, the optimal growth conditions for *B. longum* DPC 6315 in the yoghurt base were determined. The addition of 1% (w/v) yeast extract to 14% (w/v) RSM was found to promote the growth of *B. longum* DPC 6315 as it grows poorly in 14% (w/v) RSM (Fig. 4.1). Following 12, 18 and 24 h of growth in 14% (w/v) RSM with 1% (w/v) yeast extract, cell numbers of *B. longum* DPC 6315 were one log higher (>9 log CFU/ml) compared with *B. longum* DPC 6315 grown in 14% (w/v) RSM without yeast extract (Fig. 4.1). Similar findings were reported for the strain *B. breve* NCIMB 702258 by Hennessy et al. (2009), where an increase of ~ 10 fold was recorded after 43 h of anaerobic growth in 20% RSM when 20 mg/ml yeast extract was added to the growth medium compared to growth of the strain in 20% RSM with no supplementation.

The pH values recorded of the *B. longum* DPC 6315 fermentate after 6, 12, 18 and 24 h of growth in 14% (w/v) RSM with 1% yeast extract was significantly (P<0.01-0.001) lower than the pH values of *B. longum* DPC 6315 grown in 14% (w/v) RSM alone and with 0.05%, 0.1%, 0.3%, 0.5%, 0.75% (w/v) yeast extract (Fig. 4.2). There was no further significant decrease in the pH values recorded for *B. longum* DPC 6315 grown on higher levels of yeast extract.

The *B. longum* strain was added to the fermentation substrate at ~ 2×10^9 CFU/ml and by the end of fermentation no reduction in viability was observed (Fig. 4.3). Following 28 days of storage at 4ºC, the *B. longum* strain had declined to ~ 2×10^6 CFU/ml (Fig. 4.3). Furthermore, the sum of the yoghurt bacterial strains, *L. delbrueckii* ssp. *bulgaricus* and *S. thermophilus*, was above the minimum requirement of 10^7 viable

microorganisms per gram of a fermented dairy food such as yoghurt (FAO/WHO, 2003) (Fig. 4.3). In the yoghurt containing the *B. longum* strain, the viable cell numbers of *L. delbrueckii* ssp. *bulgaricus* increased from ~ 2×10^6 to 2.5×10^8 CFU/ml and in the control yoghurt increased from 5.6×10^6 to 3.6×10^8 CFU/ml (Fig. 4.3). *S. thermophilus* in the *B. longum* DPC 6315 containing yoghurt increased from 4.6×10^5 to 2.5×10^7 CFU/ml, and in the control yoghurt increased from 3.7×10^6 to ~ 6×10^7 CFU/ml. By the end of the storage period, the viable cell numbers of *L. delbrueckii* ssp. *bulgaricus* in the *B. longum* DPC 6315 containing yoghurt and the control yoghurt had declined to ~ 2×10^7 and ~ 4×10^6 CFU/ml, respectively. The viable cell numbers of *S. thermophilus* at the end of the storage period in the EPS containing yoghurt and the control yoghurt had declined to ~ 3×10^5 and to ~ 3×10^6 CFU/ml, respectively (Fig. 4.3).

4.4.2 EPS yields

When grown at 37ºC in 14% (w/v) RSM with 1% (w/v) yeast extract the EPS concentration associated with *B. longum* DPC 6315 addition was found to significantly increase ($P<0.01$) from day 0 to day 1, and to significantly increase ($P<0.001$) from day 1 to day 28 of storage at 4°C (Fig. 4.4). In the yoghurt manufacture, the amount of EPS obtained at the end of fermentation (hereafter referred to as day 0) was 36.9 (± 2.9) mg/l (Fig. 4.5), similar levels were found in reduced-fat yoghurts for EPS-producing strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (Guler-Akin *et al.*, 2009). The production of EPS increased during the first week of storage with concentrations reaching 51.4 (± 9.4) mg/l on day 7 (Fig. 4.5). After day 7 of cold storage, EPS concentrations declined with a significant decrease on day 21 ($P<0.01$) and day 28 ($P<0.01$) compared

to day 14 (Fig. 4.5). The lowest concentration of EPS in the *B. longum* DPC 6315 containing yoghurt was recorded on day 28 of cold storage at 19.3 (± 4.39) mg/l (Fig. 4.5).

4.4.3 Microstructure

The microstructure of the yoghurt samples at day 28 was then evaluated by CLSM. For both yoghurts, protein networks with large pores (black areas) (Fig. 4.6) and (Fig. 4.7) containing serum had formed during fermentation. The bacterial cells for both yoghurts were predominantly located in the pores. EPS, as indicated by staining with Wheat Germ Agglutinin Alexafluor 555, was mainly associated around the edges of the pores and most likely connected within the protein matrix in the yoghurt fermented with *B. longum* DPC 6315 (Fig. 4.6). No EPS was observed in the yoghurt fermented with CH1 starter cultures (Fig. 4.7).

4.4.4 Syneresis

In commercial manufacturing of set yoghurt syneresis is an important issue, as it can lead to the accumulation of whey (serum) on the surface of the gel, which in turn can lead to poor consumer acceptance (Ghasempour *et al*., 2012). The extent of syneresis was determined by measuring the quantity of liquid separated from yoghurt after centrifugation. The values of syneresis recorded in both the *B. longum* DPC 6315 containing yoghurt and the control yoghurt are shown in Fig. 4.8. The yoghurt manufactured with *B. longum* DPC 6315 exhibited a lower syneresis compared to the control yoghurt. It was observed that the yoghurt made using the EPS producing culture

had a significantly lower level of syneresis (P<0.05) than the control yoghurt on day 0, 1, 7 and 14 of cold storage. Moreover, the *B. longum* DPC 6315 containing yoghurt exhibited a decrease in the amount of whey separation for days 0, 1, and 7 from 63.7 (± 2.1) % to 49.7 (± 2.1) % (Fig. 4.8). During cold storage on day 14, the syneresis of the *B. longum* DPC 6315 containing yoghurt increased to 50.9 (± 2.6) % and on day 21 to 51.8 (± 2.4) %. On day 28 of cold storage, syneresis in the *B. longum* DPC 6315 containing yoghurt dropped to 49.7% (± 2.1). The control yoghurt displayed a decrease in the amount of whey separation over the 28 days of storage from 69.2 (± 3.3) % to 51.6 (± 1.3) % (Fig. 4.8). This is probably due to protein rearrangement where more protein-protein contacts are being established, which leads to improved gel strength of the control yoghurt (Isleten & Karagul-Yuceer, 2006; Ramchandran & Shah, 2009).

4.4.5 Acidifying kinetics and post-acidification

Residual acidification activity was observed for the *B. longum* DPC 6315 containing yoghurt at day 0, 1 and 7 and for the control yoghurt at day 0, 1, 7 and 14 of cold storage (Fig. 4.9). The titratable acidity, expressed as the amount of lactic acid, was significantly higher for the *B. longum* DPC 6315 containing yoghurt on day 0 (P<0.01) and day 1 (P<0.05) of cold storage. However, on day 14 (P<0.05), day 21 (P<0.05) and on day 28 (P<0.01) of cold storage the control yoghurt had a significantly higher titratable acidity than the *B. longum* DPC 6315 containing yoghurt (Fig. 4.9).

The pH of the control yoghurt was lower than that of the EPS containing yoghurt during storage indicating a slower acidity development in the yoghurt containing the EPS-producing strain. The control yoghurt displayed a significantly lower pH than the *B.*

longum DPC 6315 containing yoghurt on day 1 ($P<0.01$), day 7 ($P<0.001$), day 14 ($P<0.001$), day 21($P<0.01$) and day 28 ($P<0.01$) of cold storage (Fig. 4.10).

4.4.6 Viscosity

The texture quality of the produced yoghurt samples is expressed as viscosity of samples in millipascal seconds (mPa.S). No significant difference in viscosity between the EPS containing yoghurt and the control yoghurt was observed over 28 days of storage (Fig. 4.11).

4.5 DISCUSSION

The *Bifidobacterium* strain used in this study *B. longum* DPC 6315 displayed a ropy phenotype (Chapter 2) and was therefore chosen as an adjunct culture for yoghurt production. Previous analysis revealed that EPS production appeared to be growth associated and optimal in the presence of lactose (5% and 7% (w/v) at 37ºC (Chapter 2). No additional lactose was added to the fermentation substrate as 14% (w/v) RSM contains approximately 8% (w/v) lactose as its main sugar source. The addition of 1% (w/v) yeast extract to the fermentation medium was shown to improve the growth rate of *B. longum* DPC 6315 in this study. Yeast extract has previously been reported to improve the growth and consequently EPS production of other EPS producing cultures such as certain *S. thermophilus* strains (De Vuyst *et al*., 2003; Degeest & De Vuyst, 1999).

The effect of *in situ* produced EPS by *B. longum* DPC 6315 on starter culture activity and viability was minimal. A number of studies have reported poor growth and survival of bifidobacterial strains in fermented milk due to the poor proteolytic activities and the low pH values of these products. For instance, Hughes and Hoover, (1995) reported that the *B. longum* strain used in the fermentation of skim milk in their study showed a marked 1000 fold reduction after 7 days of storage and displayed slow growth in skim milk. Dave and Shah, (1997) also reported that the bifidobacteria strains used in their study displayed a reduction in cell counts of greater than 1000 fold when the pH of the yoghurt reached 4.5. In a study undertaken by Lamoureux et al. (2002), the *B. longum* strain used declined by more than 4 log cell counts after 28 days of storage. In general, *B. longum* strains display a poor tolerance to low pH which is commonly found in fermented food products such as yoghurt. The EPS producing *B. longum* strain used to produce

yoghurt in this study had cell counts above the FAO/WHO guidelines (FAO/WHO, 2003) after 28 days of cold storage at 4°C, albeit with a 3 log reduction in viability compared to the level at which they were inoculated.

Post-acidification, expressed as the pH value of the yoghurt samples at the end of cold storage at 4°C was significantly higher (~0.2 pH units) for the control yoghurt compared to the EPS containing yoghurt. The higher post-acidification for the control yoghurt may have resulted from organic acid production of the starter culture CH-1 whereas *B. longum* DPC 6315 in the EPS containing yoghurt used the available sugars to produce EPS rather than organic acids, leaving less available sugars for the CH-1 cultures in the EPS containing yoghurt to produce lactic acid (Ozer & Atasoy, 2002). The significant difference in pH values during cold storage between the *B. longum* DPC 6315-containing yoghurt and the control yoghurt did not appear to affect the starter cultures' viability, the viscosity, microstructure or appearance (including syneresis at end of cold storage) of the two yoghurts. Lactic acid concentrations for the EPS containing yoghurt were significantly higher than the control yoghurt on day 0 of cold storage - after this time-point the control yoghurt displayed a higher lactic acid concentration (with significantly higher values on day 21 and day 28 of cold storage) corresponding to its significantly lower pH values compared to the EPS containing yoghurt. These results suggest that the EPS produced by *B. longum* DPC 6315 is not being used by the yoghurt starter strains to produce lactic acid as the pH value is not significantly higher than the control. Lactic acid production could possibly be affected by the concentration of available nutrients (Amatayakul *et al*., 2006), and activity of the yoghurt cultures. This would explain why the control yoghurt exhibited a significantly higher lactic acid

concentration compared to the EPS containing yoghurt after day 0, as in the control yoghurt more nutrients are available for lactic acid production as they are not being used by the yoghurt cultures for EPS production.

EPS-yields were taken during cold storage for 28 days. The EPS concentration significantly increased during cold storage up to day 7 for the *B. longum* DPC 6315 containing yoghurt. It has been reported that EPS concentrations in fermented dairy products range from 40-400 mg/L (De Vuyst *et al*., 2003; Guler-Akin *et al*., 2009; Marshall & Rawson, 1999). The wide range of EPS-concentrations in different studies could be due to differences in the EPS-producing strain used for product manufacture, the level of inoculation, fermentation conditions, the isolation method, and the EPS purification and quantification methods between studies (Amatayakul *et al*., 2006). In this study *B. longum* DPC 6315 produced relatively low amounts of EPS in the yoghurt compared with the lactobacilli strains used by Kearney et al. (2011) and London et al. (submitted 2013), although this *B. longum* EPS producing strain was found to produce significantly higher concentrations of EPS when grown in 14% (w/v) RSM and 1% (w/v) yeast extract without the CH-1 yoghurt starter cultures. The levels of EPS produced in the yoghurt manufactured in this study by *B. longum* DPC 6315 correlated well with levels produced by EPS-producing strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in reduced-fat yoghurt (Guler-Akin *et al.*, 2009). The decrease in EPS levels after day 7 of cold storage in the yoghurt made with *B. longum* DPC 6315, is in contrast to that seen in the study of Prasanna et al. (2013), where EPS levels produced in two separate low fat set yoghurts made with two different *Bifidobacterium* strains, one a *B. longum* and the other a *B. infantis* strain were not found to change significantly over 28 days of cold

storage at 4ºC. With prolonged storage a decrease in EPS-concentrations for the EPS-containing yoghurt was reported by Purwandari et al. (2007), where the EPS concentrations in all batches of yoghurt made with two different strains of *Streptococcus thermophilus* declined substantially ($P<0.05$), especially in the first week. The significant ($P<0.05$) reduction of the EPS concentration reported in these yoghurt batches during the first weeks of cold storage could be due to the activity of enzymes capable of degrading EPS (Degeest *et al*., 2002). It is possible that EPS degradation may be activated due to the cell's requirements for energy maintenance, as the EPS may serve as an energy store in the presence of an excess of nutrients, which may be used later in the cell metabolism (Tolstoguzov, 2003). Many studies have demonstrated the reduction in EPS concentrations after prolonged incubation of *S. thermophilus* (Cerning, 1990; Cerning *et al*., 1988; De, 1998; De Vuyst, 1999; Gancel & Novel, 1994; Macura & Townsley, 1984; Pham *et al*., 2000). It has been suggested that EPS degradation during fermentation is possibly due to a change in the physical or chemical factors linked to glycosylhydrolase activity. In the study performed by Pham et al. (2000), a greater reduction in EPS yields was observed in the case of lactose-grown *Lactobacillus rhamnosus* R cells than in glucose-grown cells. It has been suggested by Cerning et al. (1988), that EPS degradation is due to an enzyme, possibly a glycohydrolase, which gradually leads to the destruction of the polymer. The only clear evidence of the existence and effect of glycosylhydrolases (degrading heteropolysaccharides from LAB) has been shown in studies performed using the strains *L. rhamnosus* R (Pham *et al*., 2000) and *S. thermophilus* LY03 (Degeest *et al*., 2002). Pham et al. (2000) suggested that the slow rate of reduction in viscosity of EPS-containing media is due to the mode of enzymatic action involving exotype mechanisms,

which progressively split glycosidic linkages leading to polymers with lower molecular masses. In their study, it was reported that there was up to 82% reduction in EPS quantity if the EPS producing *L. rhamnosus* R strain was incubated for up to 48 h. It is likely that this EPS degradation is also taking place in the EPS containing yoghurt produced in this study as no reduction in EPS concentration was observed when *B. longum* DPC 6315 was grown in 14% (w/v) RSM and 1% (w/v) yeast extract without the addition of the CH-1 starter cultures. Glycosylhydrolases released from cells of the *S. thermophilus* yoghurt starter strain used in this study could potentially be acting on the EPS produced by *B. longum* DPC 6315 leading to EPS degradation.

The yoghurt made with the EPS producing culture had a positive effect in reducing syneresis on day 0, 1, 7 and 14 of cold storage without significant difference in syneresis between the control and EPS containing yoghurt on days 21 and 28. This is possibly due to the degradation of EPS in the EPS containing yoghurt with a reduction of EPS on day 14, 21 and 28 of cold storage, respectively. Syneresis is considered a major defect in the yoghurt industry and is directly related to the extent of physical disturbance to the network of protein micelles (Tamime & Robinson, 2007a). It has been previously reported that ropy EPS has a greater ability to retain serum, resulting in lower syneresis (Folkenberg *et al*., 2005; Lucey *et al*., 1998). Some other studies have previously reported that the intensity of syneresis decreased in set-type yoghurts during prolonged storage (Guzel-Seydim *et al*., 2005; Kearney *et al*., 2009; Purwandari *et al*., 2007; Robitaille *et al*., 2009). The EPS containing yoghurt in this study did show a significant reduction in the amount of syneresis up to day 14 of storage compared to the control yoghurt, which demonstrates that at a certain level the EPS produced by *B. longum* DPC

6315 has the ability to bind free water and reduce syneresis in yoghurt. It is possible that this EPS-producing strain could have potential in reducing syneresis levels in commercial yoghurt production if a manufacturing process is found that prevents its EPS degradation.

The microstructural analysis of the *B. longum* DPC 6315 containing yoghurt reveals that the EPS was well distributed in the product and was generally located around the edges of the pores. A similar microstructure was also observed in the studies performed by Folkenberg et al. (2005) and Kearney et al. (2009), where an overlap of protein and EPS was observed and the yoghurt product was described as being very shiny, very ropy, with low serum separation and being resistant to mechanical treatment, which correlates positively with the results of this study. The extent of the interaction between the EPS produced by *B. longum* DPC 6315 and the yoghurt network is unknown, however the association between EPS and protein appears to be positive, as incompatibility between EPS and protein would lead to phase separation, with EPS being expelled into the serum phase (Hassan *et al*., 2003a).

It was visually observed that both the *B. longum* DPC 6315 containing and control yoghurts were slightly ropy and shiny. No significant difference between the viscosity of the *B. longum* DPC 6315 containing yoghurt and the control yoghurt was found. This was possibly due to the amount of EPS present in the yoghurt being too low to influence viscosity, although previously in other studies no clear relationship between EPS production yields and viscosity in yoghurt and fermented milks have been observed (Bouzar *et al*., 1997; De Vuyst *et al*., 2003; Wacher-Rodarte *et al*., 1993). It is also possible that the type of EPS produced by *B. longum* DPC 6315, its molecular mass,

charge, radius of gyration and polymer stiffness might not be conductive to increased viscosity in yoghurt.

Overall, this study demonstrated that the production of EPS by *B. longum* DPC 6315 in yoghurt reduced the syneresis of the yoghurt up to day 14 of cold storage. Not surprisingly, the presence of this strain in the yoghurt led to significantly higher levels of EPS compared to the control yoghurt up to day 28 of cold storage. The levels of EPS did significantly decline after day 7 of cold storage which suggests the presence of EPS degrading enzymes possibly originating from the yoghurt starters. The EPS producing bifidobacterial strain used to manufacture yoghurt in this study did survive to the FAO/WHO minimum required levels of a probiotic culture in a fermented food such as yoghurt after 28 days of cold storage. However, it is important to emphasize that this is a general level and not one which is supported by clinical studies with dose response levels for each particular strain. The EPS produced in the *B. longum* DPC 6315 containing yoghurt did not significantly increase the viscosity of this yoghurt compared to the control, although both yoghurts displayed a shiny, slightly ropy appearance. These results suggest that addition of the adjunct strain offers some technological improvements to the product over and above its probiotic characteristics.

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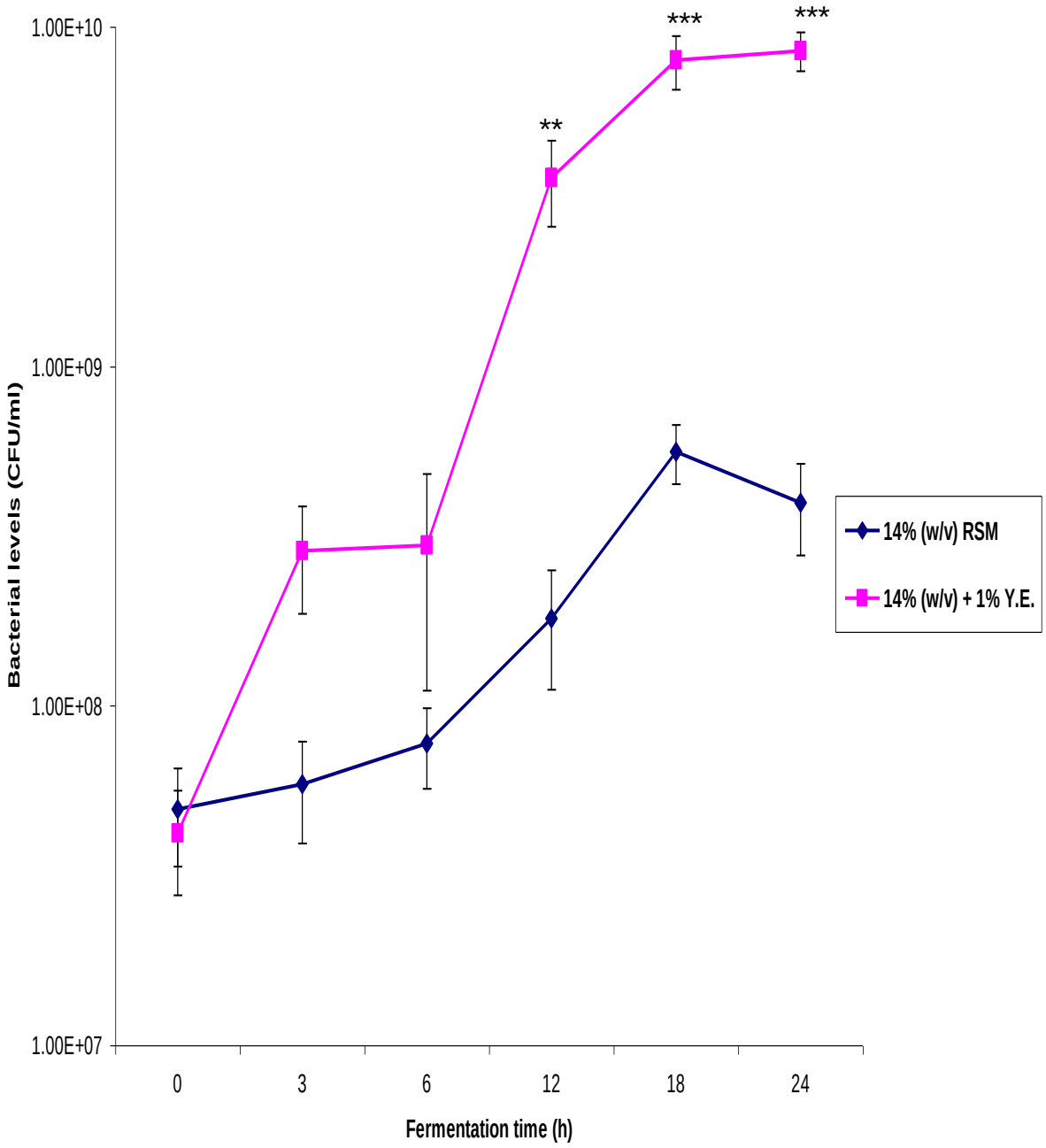

Figure 4.1 Growth of *B. longum* DPC 6315 at 37°C in 14% (w/v) RSM and 14% RSM with 1% (w/v) yeast extract (Y.E.) for 24 h under anaerobic conditions.

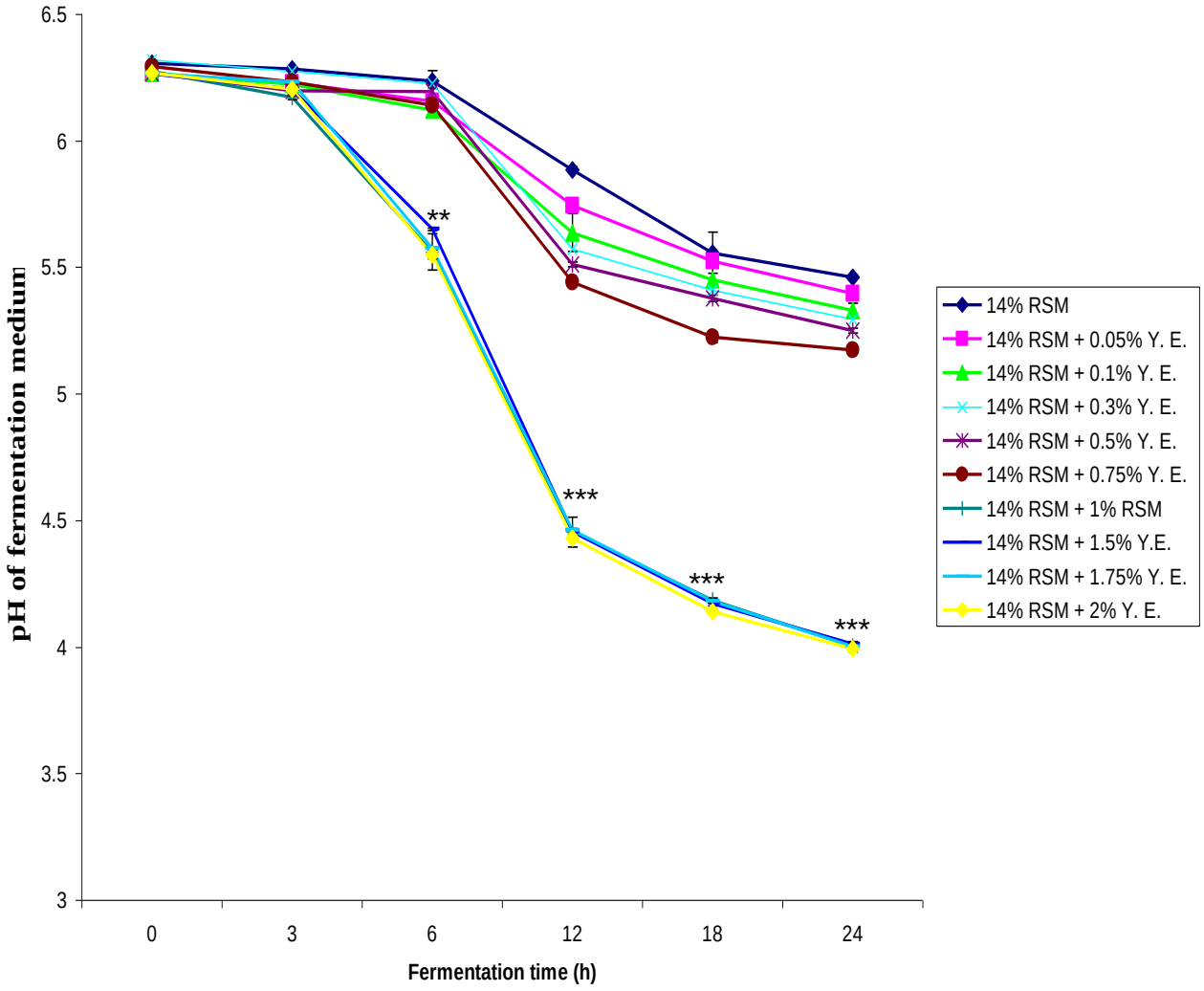

Figure 4.2 pH values of *B. longum* DPC 6315 grown at 37º in 14% (w/v) RSM with different concentrations of Y.E. (0%-2%) for 24 h under anaerobic conditions.

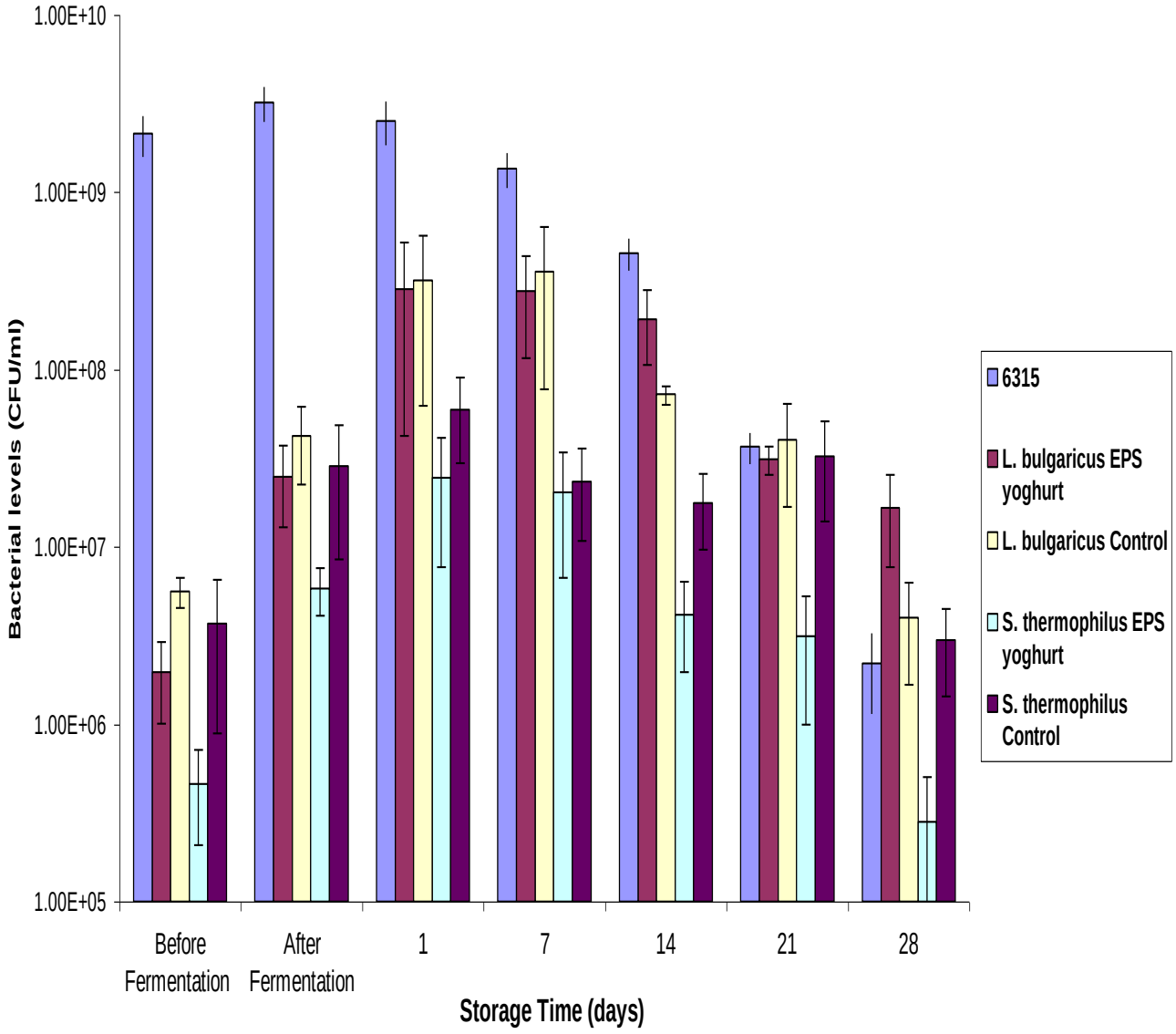

Figure 4.3 Survival of *B. longum* DPC 6315 and CH-1 yoghurt starter cultures during yoghurt manufacture and over 28 days of cold storage.
Fermentation = 37ºC under anaerobic conditions for 6.5 h. Storage temperature = 4 ºC.

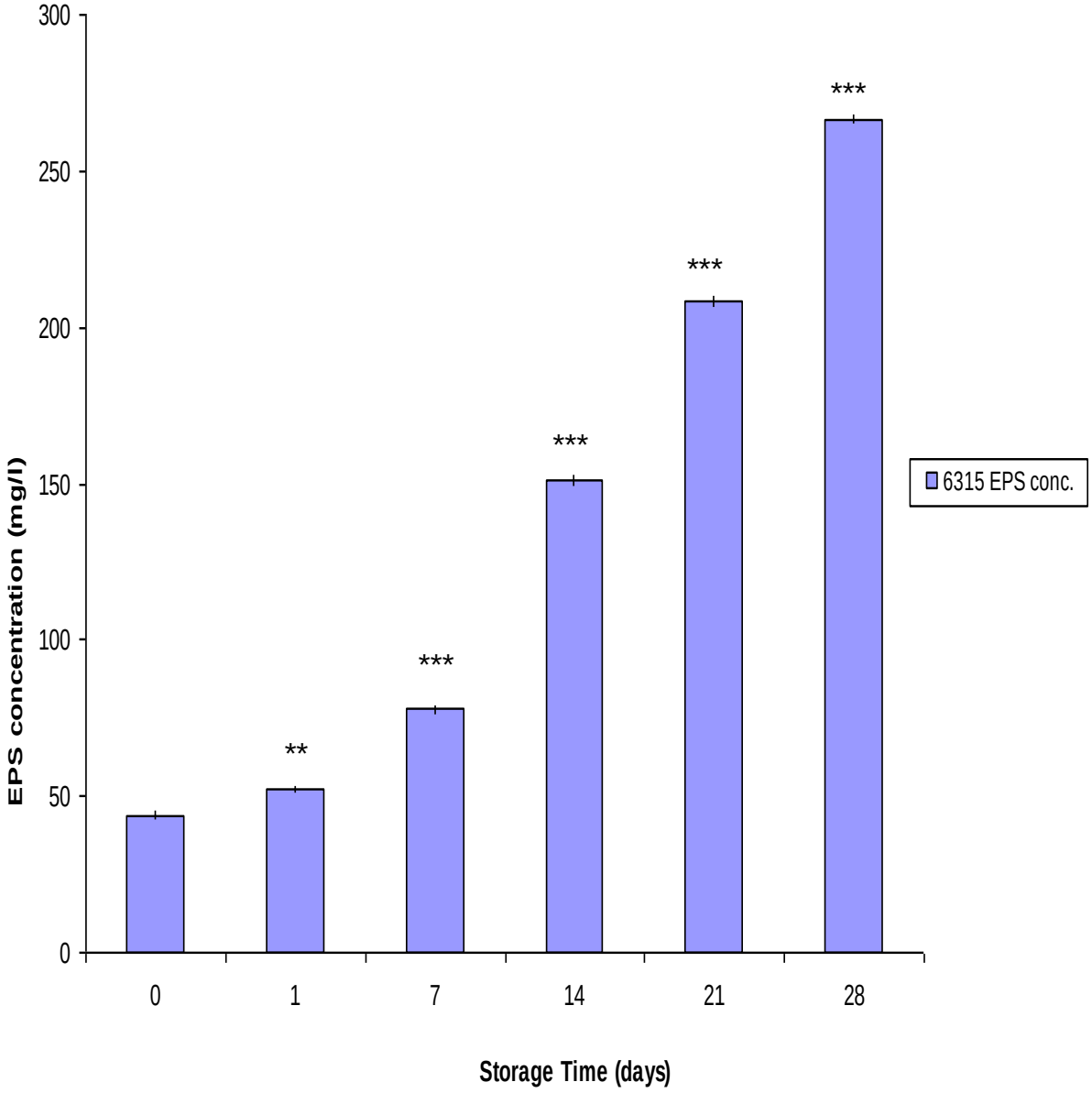

Figure 4.4 EPS concentrations of *B. longum* DPC 6315 grown at 37°C in 14% (w/v) RSM with 1% (w/v) yeast extract during cold storage at 4°C over 28 days.

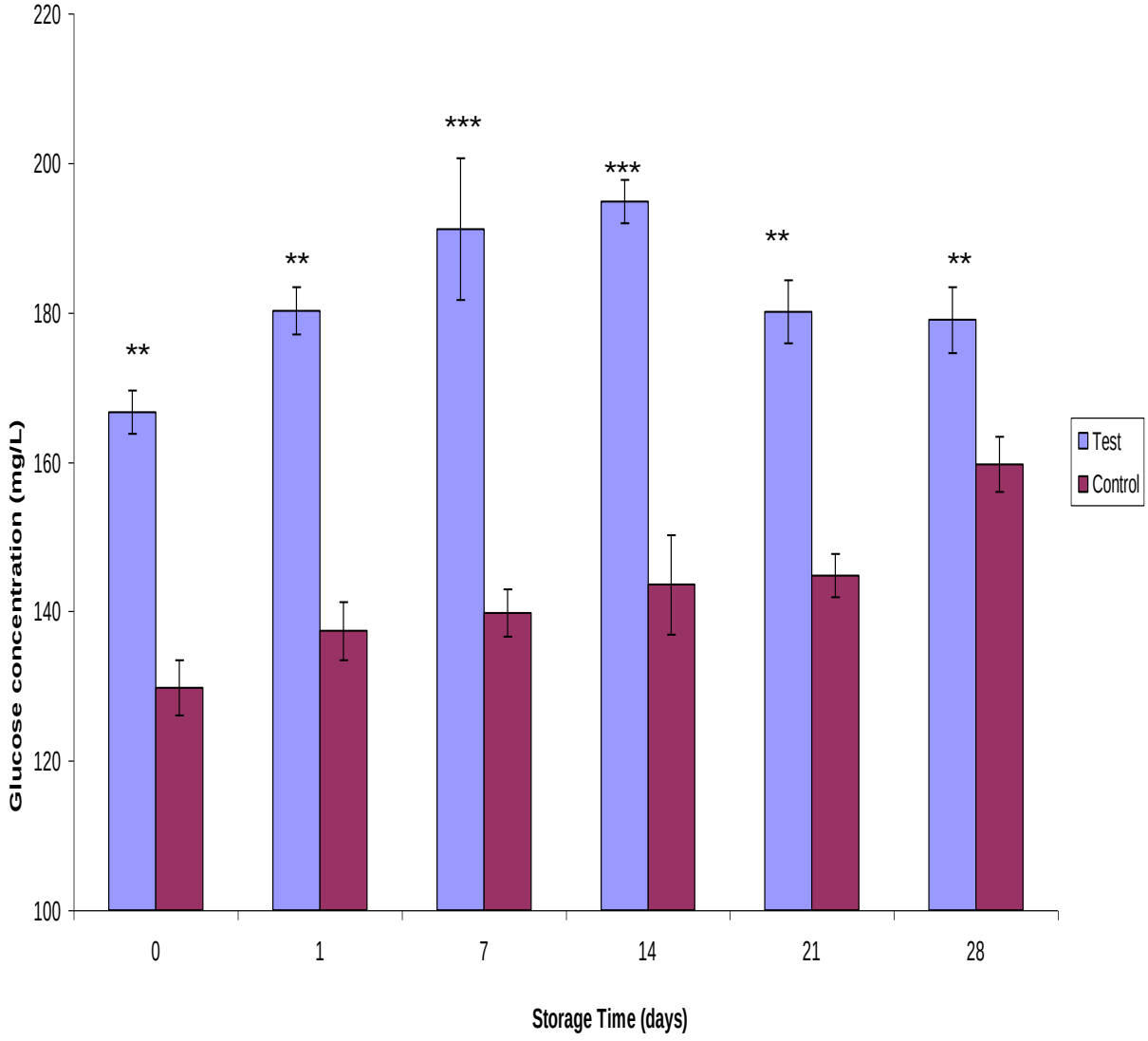

Figure 4.5 Glucose concentrations of *B. longum* DPC 6315 containing and control yoghurt over 28 days of cold storage.

EPS concentrations of *B. longum* DPC 6315 containing yoghurt = Test yoghurt glucose concentration – Control yoghurt glucose concentration.

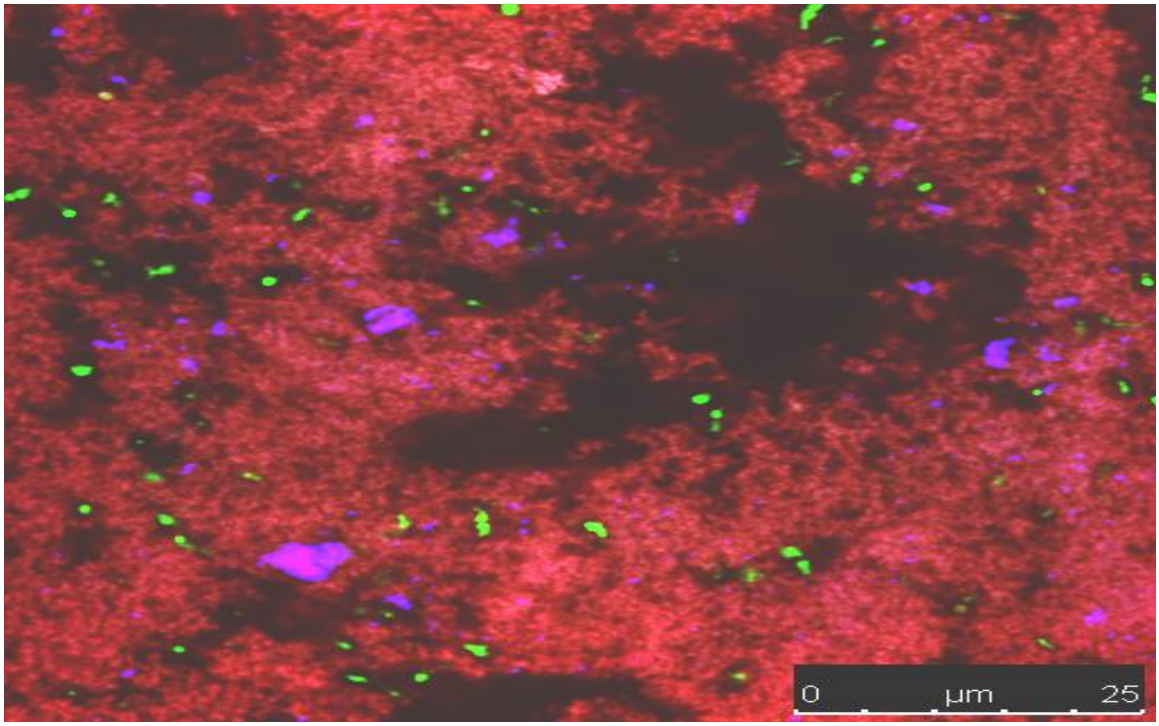

Figure 4.6 Confocal laser scanning microscope (CLSM) image of the *B. longum* DPC 6315 containing yoghurt at 28 days of cold storage. The bacteria, the EPS, and the protein aggregates were labeled with Syto 9, Wheat Germ Agglutinin Alexa Fluor 555 and Fast Green FCF, respectively. Therefore, the bacteria appear green, the protein network appears red and the EPS fluoresces blue. The yoghurt pores appear as black in the image.

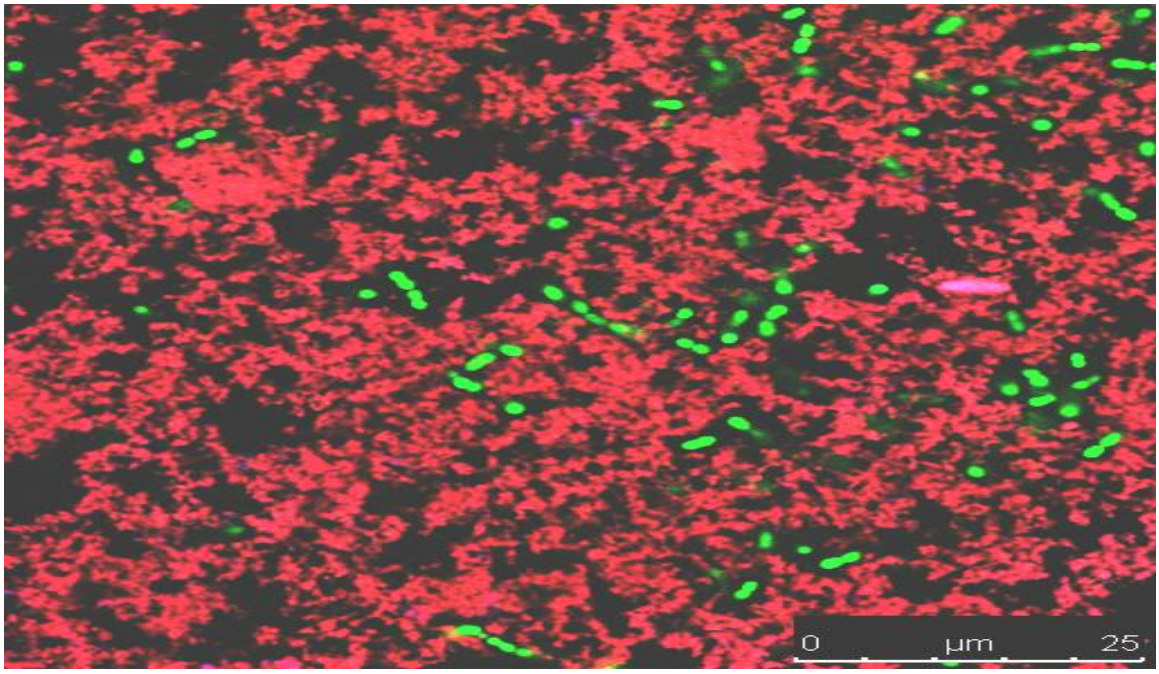

Figure 4.7 CLSM image of the control yoghurt at 28 days of cold storage. The bacteria and the protein aggregates were labeled with Syto 9 and Fast Green FCF, respectively. Therefore, the bacteria appear green and the protein network appears red. The yoghurt pores appear as black in the image.

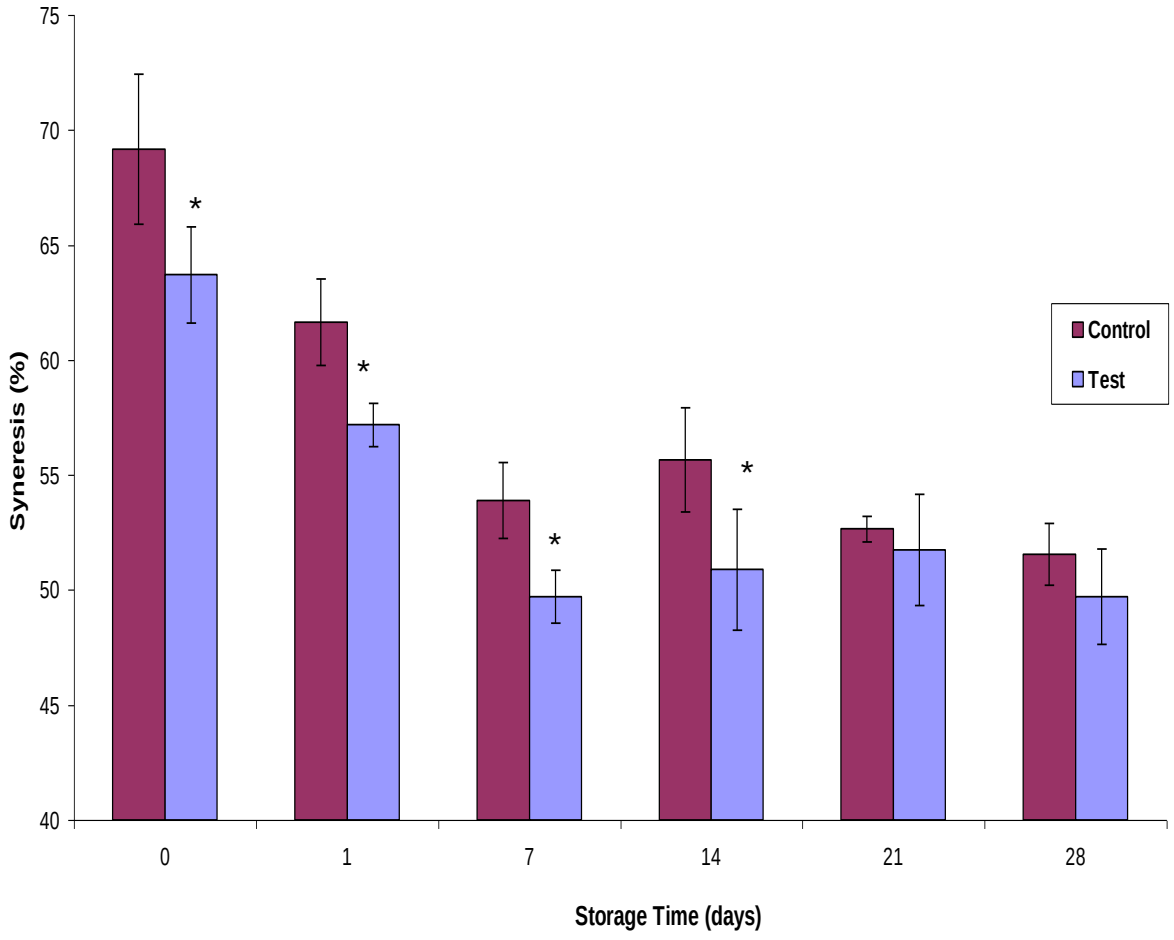

Figure 4.8 Percentage of syneresis of *B. longum* DPC 6315 containing and control yoghurt over 28 days of cold storage.

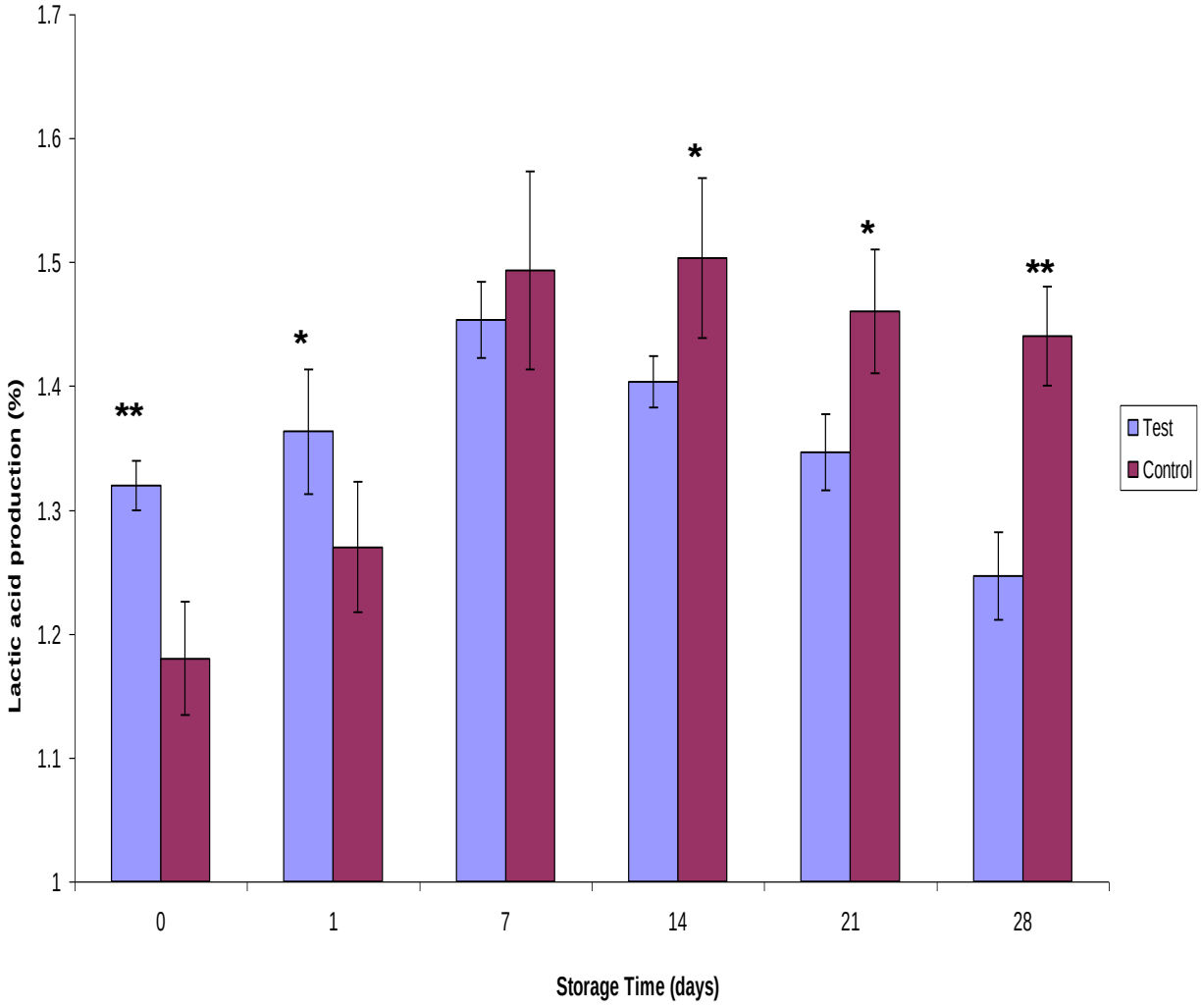

Figure 4.9 Percentage lactic acid concentration of *B. longum* DPC 6315 containing and control yoghurt over 28 days of cold storage.

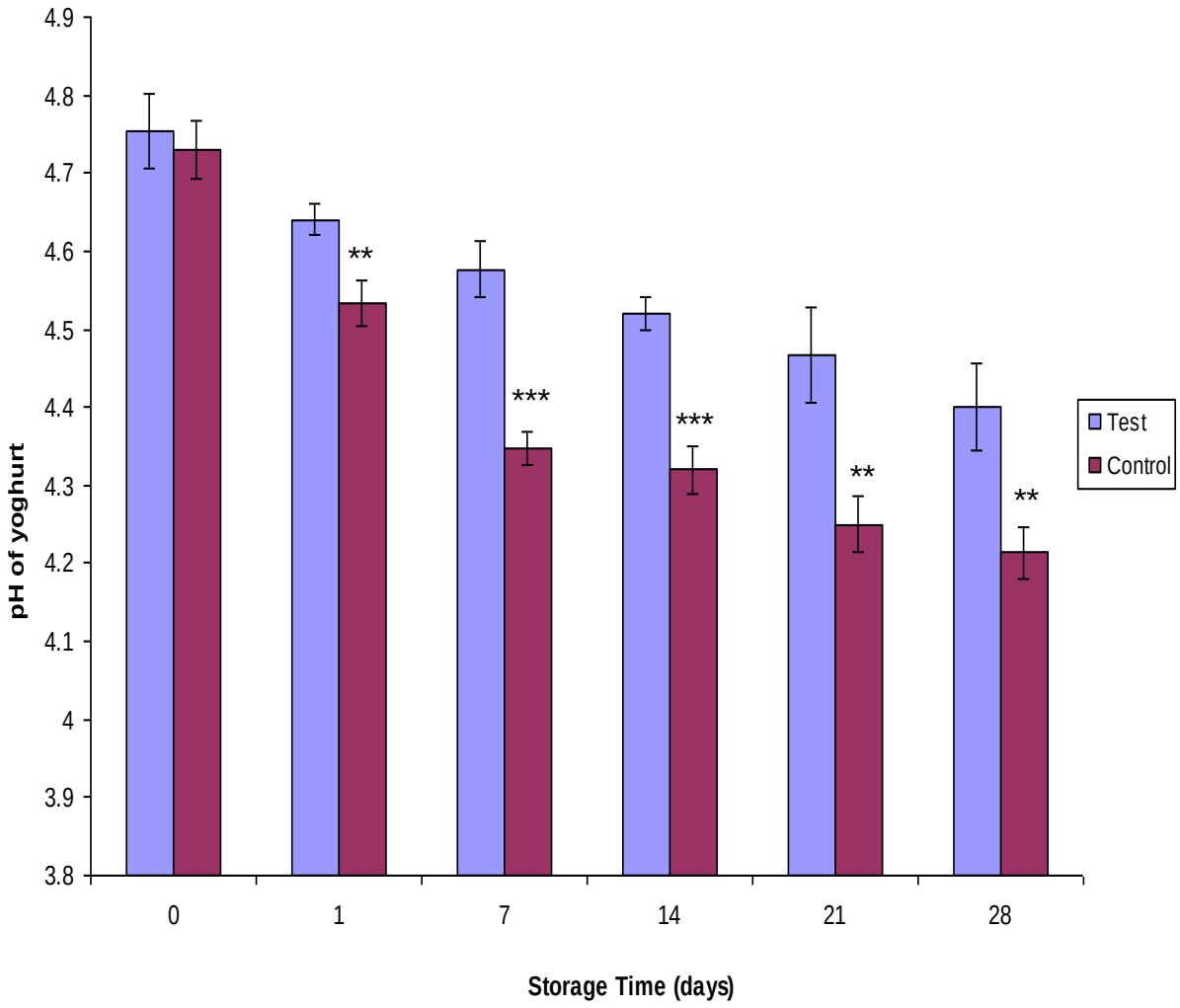

Figure 4.10 pH of *B. longum* DPC 6315 containing and control yoghurt over 28 days of cold storage.

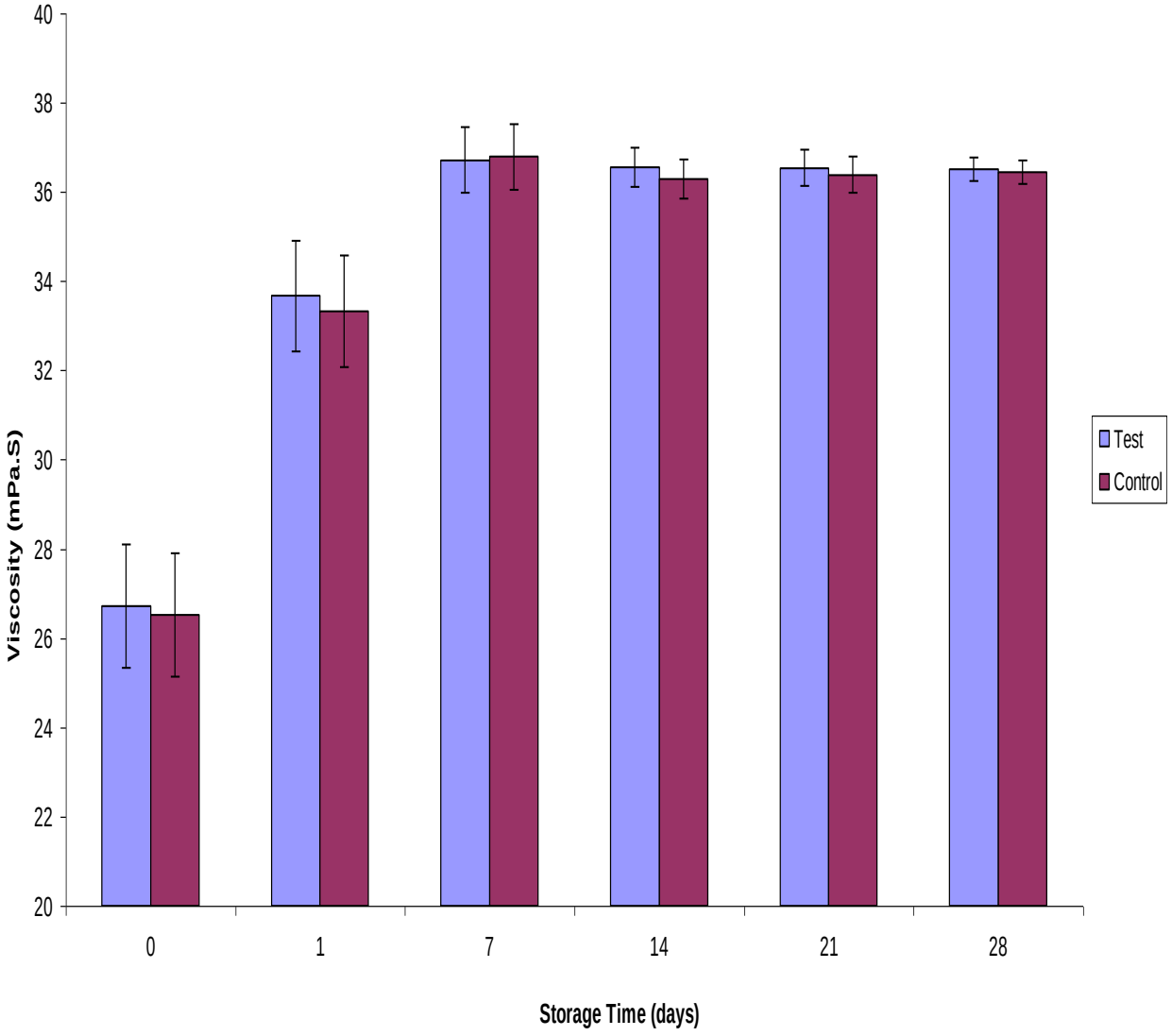

Figure 4.11 Viscosity of *B. longum* DPC 6315 containing and control yoghurt over 28 days of cold storage.

CHAPTER 5

Effect of *Bifidobacterium longum* DPC 6315 ingestion on fatty acid metabolism and intestinal microbiota in a murine model.

5.1 ABSTRACT

B. longum DPC 6315 has previously been found to be an efficient producer of EPS (Chapter 2), CLA (Barrett *et al.*, 2007), possess the *lanA* gene (Chapter 2) and a lantibiotic operon (Chapter 3). In this study, *B. longum* DPC 6315 was fed along with a high fat diet enriched with α-linolenic acid (test group) to BALB/c mice. In the control or unsupplemented group, BALB/c mice were fed with the high fat diet enriched with α-linolenic acid but without the strain. *B. longum* DPC 6315 was found to survive passage through the mouse GIT. Following 6 weeks of feeding *B. longum* DPC 6315, it was recovered at ~4.4×10^4 CFU/g faeces in the test group. Fatty acid composition analysis revealed that mice that received *B. longum* DPC 6315 had significantly lower ($P<0.01$) levels of linoleic acid and γ-linolenic acid ($P<0.001$) acid in the liver, and stearic acid ($P<0.05$) levels in the brain compared with unsupplemented mice. In contrast, mice that received *B. longum* DPC 6315 had significantly higher concentrations of stearic acid ($P<0.05$), EPA, ($P<0.05$) and DPA, ($P<0.05$) in the liver. The microbial diversity of the caecal samples from the two mouse groups was determined using 16S rRNA amplicon pyrosequencing technology. In total 425,285 sequences were obtained from 19 caecal samples collected from the two mouse groups. The mouse microbiota samples taken from both groups fed *B. longum* DPC 6315 and α-linolenic acid and the unsupplemented group were dominated by *Firmicutes* (72% and 61% respectively), followed by *Bacteroidetes* (17% and 29% respectively). *Firmicutes* were found at a significantly ($P=0.0076$) higher (0.2 fold increase) relative abundance in the group that received *B. longum* DPC 6315, while *Bacteroidetes* were found at a significantly ($P=0.0057$) higher (0.7 fold increase) proportions in the unsupplemented group. At the genus level, *Alistipes* was detected at a

significantly (P=0.04) higher (0.8 fold increase) relative abundance in the unsupplemented group, while *Roseburia* was detected at a significantly (P=0.04) higher (0.5 fold increase) relative abundance in the group that received *B. longum* DPC 6315. In conclusion, supplementation with *B. longum* DPC 6315 led to significant differences in fatty acid composition in both the murine liver and brain tissues compared to supplementation with α-linolenic acid alone. *B. longum* DPC 6315 supplementation also led to significant differences in caecal microbiota composition at both the phylum and genus level.

5.2 INTRODUCTION

It is estimated that the healthy human adult GIT contains approximately 10^{14} microorganisms (Vaughan *et al*., 2000; Zboril, 2002). These microbial cells outnumber host cells by a factor of 10, and contain at least 100-fold more genes than the human genome. This microbial community is thought to play a key role in providing regulatory signals for immune and gastrointestinal maturation in early life (O'Hara & Shanahan, 2006; Round & Mazmanian, 2009; Shanahan, 2009). The collective genome of these microorganisms (the metagenome) supplies a wide range of metabolic and biochemical functions that otherwise could not be performed by the host (Ley *et al.*, 2006). These functions include metabolism (i.e. CLA, SCFA and bacteriocin production and prebiotic utilisation) epithelial development, immune modulation, regulation of fat storage and protection against pathogens (Arunachalam *et al.*, 2000; Backhed *et al.*, 2004; Claus *et al.*, 2008; Corr *et al.*, 2007; Wang *et al.*, 2004; Wells *et al.*, 2010). The dominant populations present in the adult microbiotia are *Firmicutes* (including genera of *Clostridia* and LAB) and *Bacteriodetes*, while *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* being present to a lesser extent (Eckburg *et al*., 2005).

Bifidobacteria are naturally found in the human GIT and are present in particularly higher numbers in early infancy (Chierici *et al.*, 2003), and in breast-fed infants where they can constitute >50% of the intestinal microbiota (Yoshioka, 1991). The presence of bifidobacteria throughout life in the human GIT and their metabolic activities are considered integral to the maintenance of human health and well being (Van der Werf & Venema, 2001). Bifidobacteria are used globally as probiotics (‘live microorganisms,

which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002) in many food products such as yoghurt, milk, infant formula, cheese, and dietary supplements (Champagne *et al.*, 2005; Charalampopoulos *et al.*, 2002; Mattila-Sandholm *et al.*, 2002; Phillips *et al.*, 2006; Vinderola *et al.*, 2000).

Numerous strains of bifidobacteria such as *B. longum*, *B. animalis*, *B. adolescentis* and *B. pseudocatenulatum* produce exopolysaccharides (Ruas-Madiedo *et al.*, 2007). EPS isolated from bifidobacteria have been found to possess prebiotic, immune stimulatory and anti-microbial properties as described below. Salazar et al. (2008) found that some EPS produced by human intestinal bifidobacteria possessed prebiotic abilities on other bifidobacteria when tested in a non-pH controlled faecal batch fermentation. This prebiotic effect was seen as an increase in the levels of bifidobacteria comparable to the prebiotic inulin, this effect was observed with EPS isolated from 11 different strains of bifidobacteria, which included 2 *B. animalis* strains, 5 *B. pseudocatenulatum* strains and 4 *B. longum* strains after 1 or 5 days of incubation.

Furthermore, a shift in the short chain fatty acid profile of the faecal cultures was observed after incubation with the EPS isolated from the 11 bifidobacteria strains tested, causing a decrease in the molar proportions of acetic acid, an increase in propionic acid levels, decreases or moderate increases in butyric levels, and a reduction in the acetic acid-to-propionic acid ratio with time (Salazar *et al.*, 2008). The reduction in the acetic acid-to-propionic acid ratio has been proposed as a potential indicator of the hypolipidemic effect (inhibition of cholesterol and fatty acid biosynthesis in the liver, resulting in a decrease in lipid levels in blood) of prebiotics (Delzenne & Kok, 2001).

Salazar et al. (2009) also performed a study using two EPS's one isolated from *B. longum* subsp. *longum* IPLA E44 from the study (Salazar *et al*., 2008) and one from *B. animalis* subsp. *lactis* IPLA R1 to test their prebiotic effects in pH-controlled batch culture fermentation. It was reported that both the EPS's tested in this study promoted a rapid increase in the amount of acetic and lactic acids during the early stages of fermentation (until 10 h and in some cases until 24 h of fermentation) and of total bacteria during fermentation. Fluorescent in situ hybridization analysis clearly showed that both the EPS tested stimulated the growth of *Lactobacillus/Enterococcus* and the genus *Bifidobacterium*, which was also reported by this group in their previous study (Salazar *et al*., 2008). Wu et al. (2010) reported that EPS isolated from a *B. longum* strain tested in their study stimulated growth and induced IL-10 secretion in the murine macrophage/monocyte-like cell line J774A.1 cells when exposed to lipopolysaccharide (LPS). EPS treatment also induced lower levels of TNF-α secretion by LPS and decreased the LPS-induced morphological changes in J774A.1 cells. The EPS isolated from this particular *B. longum* strain was also found to inhibit seven different bacterial pathogens and food spoilage bacteria such as a particular strain of *E. coli*, *S. typhimurium*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *S. aureus*, *Bacillus subtilis and Bacillus cereus. P. aeruginosa* and *V. parahaemolyticus* were not inhibited by 20 ppm (µg/ml) EPS treatment but all 7 of the tested pathogens and food spoilage bacteria were inhibited by 80 ppm (µg/ml) EPS, although EPS did not inhibit *P. aeruginosa* effectively.

Bacterially-produced antimicrobial peptides are called bacteriocins. Two major classes of gram-positive bacteriocins have been recognized, Class I undergo significant

post-translational modifications while Class II are unmodified (Cotter *et al.*, 2005; Rea *et al.*, 2011). Lantibiotics which are small peptides containing internal bridges resulting from the formation of (β-methyl) lanthionine residues make up the majority of Class I bacteriocins. The first lantibiotic produced by a *Bifidobacterium* species was identified by Lee et al. (2008) in the strain *B. longum* subsp. *longum* DJO10A.

This lantibiotic operon was found to contain all the genes necessary for lantibiotic production including the *lanA* gene which encodes the prepeptide involved in lantibiotic production (Lee *et al.*, 2011). It was reported that lantibiotic production by *B. longum* subsp. *longum* DJO10A only occurred on agar media and not in broth media unless an inducer such as the purified lantibiotic itself was first added to the broth media (Lee *et al*., 2011). The lantibiotic produced by *B. longum* subsp. *longum* DJO10A was collected from agar cultures and purified and it was found to possess a broad spectrum of inhibition activity against various gram-positive and gram-negative bacteria. Lantibiotics can confer a selective advantage to the producer organism by inhibiting the growth of other organisms found in the same habitat. Lantibiotics have the potential to be used as natural food preservatives due to their high stability and broad inhibition spectrum, e.g. Nisin (Chen & Hoover, 2003; Deegan *et al.*, 2006).

This study investigated the possible effects of feeding *B. longum* DPC 6315 on the fatty acid composition of the murine liver and brain, and its effect on the overall microbiota of the mouse caecal contents *in vivo* using 454 pyrosequencing analysis. The caecum was chosen as the gut habitat for sampling because it is an anatomically distinct structure, situated between the distal small intestine and colon, and is colonized with high quantities of a readily harvested microbiotia for metagenomic analysis (Turnbaugh *et al*.,

2006). As such the microbiota from this site may provide a better representation of microbiota than from faecal samples.

5.3 MATERIALS AND METHODS

5.3.1 Animals and treatment

Female BALB/c mice were purchased from Harlan (Briester, Oxon, UK) at 8 weeks of age and housed under barrier-maintained conditions within the Biological Services Unit, University College Cork (UCC). All animal experiments were approved by the UCC Animal Ethics Committee, and experimental procedures were conducted under the appropriate license from the Irish Government. Mice were allowed to acclimatize for 1 week before the start of the study and were fed *ad libitum* with Teklad Global rodent standard diet (Harlan Laboratories, Madison, WI, USA #2018S) and allowed free access to water at all times. Mice were housed in groups of 5 per cage and kept in a controlled environment at 25°C under a 12-h-light/12-h-dark cycle. After 1 week of acclimatization, the mice were divided into 2 groups (n =10/group): a group fed flaxseed-oil enriched diet and *B. longum* DPC 6315 (approximate daily dose of 10^9 microorganisms) and a group fed flaxseed-oil enriched diet and placebo freeze-dried powder (15% w/v trehalose in dH_2O). The flaxseed-oil enriched diet contained the following nutrient composition: corn starch (32.45%), casein (20.0%) sucrose (15%), maltodextrin (12.0%), cellulose (5.0%), L-cysteine (0.3%), choline bitartrate (0.25%), TBHQ antioxidant (0.002%), mineral mix (3.5%) and vitamin mix (1.5%) and the following composition of fat: flaxseed oil (5.5%), palm oil (1.5%), safflower oil (1.5%), olive oil (1.5%). Body weight and food intake were assessed weekly. After 6 weeks on the experimental diets, the animals were killed by decapitation. Liver, brain, fat pads (epididymal, perirenal, and mesenteric), GIT from stomach to anus, and caecal contents

were removed, blotted dry on filter paper, weighed, and flash-frozen immediately in liquid nitrogen. All samples were stored at -80°C until processed.

5.3.2 Preparation and administration of *B. longum* DPC 6315

Rifampicin-resistant variants of *B. longum* DPC 6315 were isolated by spread-plating ~10^9 CFU from an overnight culture onto mMRS agar (Difco) supplemented with 0.05% (w/v) L-cysteine hydrochloride (98% pure; Sigma) containing 500 µg rifampicin/ml (Sigma). After anaerobic incubation (anaerobic jars with Anaerocult A gas packs; Merck) at 37°C for 3 days, colonies were stocked in mMRS broth containing 40% (v/v) glycerol and stored at -80°C. To confirm that the rifampicin-resistant variant was identical to the parent strain, PFGE was used for molecular fingerprinting.

Before freeze-drying, *B. longum* DPC 6315 was grown in mMRS by incubating overnight at 37°C under anaerobic conditions. The culture was washed twice in phosphate-buffered saline and resuspended at a concentration of ~2×10^{10} cells/ml in 15% (w/v) trehalose (Sigma) in dH_2O. One-milliliter aliquots were freeze-dried by using a 24-h program (freeze temperature, -40°C; condenser set point, -60°C; vacuum set point, 600 mTorr). Each mouse that received the bacterial strain consumed ~1×10^9 live microorganisms/day. This was achieved by resuspending appropriate quantities of freeze-dried powder in water, which mice consumed ad libitum. Mice that did not receive the bacterial strains received placebo freeze-dried powder [15% (w/v) trehalose in dH_2O]. Water containing either the bacterial strains or placebo freeze-dried powder was the only water supply provided to the animals throughout the trial. Freeze-dried powders with the bacterial strains underwent continuous quality control of cell counts for the duration of

the trial by plating serial dilutions on MRS agar supplemented with 100 µg mupriocin/ml (Oxoid) and 100 µg rifampicin/ml (Sigma) and incubating plates anaerobically for 72 h at 37°C.

5.3.3 Culture-dependent microbial analysis

Fresh faecal samples were taken from BALB/c mice every second week for microbial analysis. Microbial analysis of the faecal samples involved enumeration of the *B. longum* DPC 6315 strain by plating serial dilutions on MRS agar supplemented with 100 µg mupriocin (Oxoid)/ml, 100 µg rifampicin/ml (Sigma), and 50 U nystatin/ml at 37°C. Agar plates were incubated anaerobically for 72 h at 37°C.

5.3.4 Lipid extraction and fatty acid analysis

Lipids were extracted with chloroform:methanol (2:1, v/v; Fisher Scientific, Dublin, Ireland) according to the method of (Folch *et al.*, 1957). Fatty acid methyl esters (FAMEs) were prepared by using first adding 10 ml 0.5 N NaOH (Sigma) in methanol for 10 min at 90°C followed by 10 ml 14% BF_3 in methanol (Sigma) for 10 min at 90°C (Park & Goins, 1994). FAMEs were recovered with hexane (Fisher Scientific). Before gas-liquid chromatographic analysis, samples were dried over 0.5 g anhydrous sodium sulphate (Sigma) for 1 h and stored at -20°C. FAMEs were separated by gas-liquid chromatography (Varian 3800; Varian, CA, USA) fitted with a flame-ionization detector by using a Chrompack CP Sil 88 column (Chrompack; 100 m × 0.25 mm internal diameter, 0.20-µm film thickness) and helium as the carrier gas. The column oven was programmed to be held initially at 80°C for 8 min and then increased by 8.5°C/min to a

final column temperature of 200°C. The injection volume used was 0.6 µl, with automatic sample injection on a SPI 1093 splitless on-column temperature-programmable injector. Peaks were integrated by using the Varian Star chromatography Workstation version 6.0 software, and peaks were identified by comparison of retention times with pure FAME standards (Nu-Chek Prep, Minnesota, USA). The percentage of individual fatty acids was calculated according to the peak areas relative to the total area (total fatty acids were set at 100%). All fatty acid results are shown as means ± SEMs in g/100 g FAME.

5.3.5 Generation of 16S rRNA amplicons for high-throughput sequencing

The generation of 16S rRNA amplicons was performed as described previously (O' Sullivan *et al.*, 2011). DNA was extracted from samples according to a previously described protocol (Zoetendal *et al.*, 2006) using the QIAamp DNA stool Mini kit (Qiagen) with the addition of a bead beating step. Universal 16S rRNA primers, as previously described (Claesson *et al.*, 2009), were used to amplify from highly conserved regions corresponding to those flanking the V4 region. Pyrosequencing was performed at the Teagasc Moorepark 454 Sequencing facility on a Genome Sequencer FLX platform (Roche Diagnostics Ltd, West Sussex, UK) according to the manufacturer's protocols.

5.3.6 Bioinformatic analysis

Resulting raw sequence reads were quality trimmed as previously described (Claesson *et al*., 2009). Trimmed FASTA sequences were then BLASTed (Altschul *et al*., 1997) against a locally installed version of SILVA 16S rRNA database (Pruesse *et al.*,

2007), with the top 50 hits against the database selected using default parameters. The taxonomic distribution of reads was determined using MEGAN (Huson *et al*., 2007a), with modified accession look-up tables for mapping the SILVA assignments to NCBI taxonomy. MEGAN assigns reads to NCBI taxonomies by using the Lowest Common Ancestor algorithm. Bit scores were used from within MEGAN for filtering the results before tree construction and summarization. A bit score of 86 was selected as previously used for 16S ribosomal sequence data (Urich *et al*., 2008). Phylum and family counts for each subject were extracted from MEGAN. Sequence reads were clustered into operational taxonomical units (OTU's) using the QIIME suite of software tools (Caporaso *et al.*, 2010), and OTU's were aligned and chimeric OTU's were removed using the ChimeraSlayer (Haas *et al.*, 2011) program. A phylogenetic tree was generated using the FastTreeMP (Price *et al.*, 2010) tool. Subsequently alpha and beta diversities of the samples were calculated. Principal coordinate analysis (PCoA) and hierarchial clustering of samples were implanted; PCoA plots were visualized with the KiNG viewer (Chen *et al.*, 2009)) and unweighted pair group method with arithmetric mean (UPGMA) plots were visualized using DENDROSCOPE (Huson *et al*., 2007b).

5.3.7 Statistical analysis

Results in the text, tables, and figures are presented as means ± SEMs (per group). To investigate the effect of dietary supplementation with *B. longum* DPC 6315 on host tissue fatty acid composition between the two groups, data was analysed using unpaired T-test.

To assess whether differences in microbiota composition between groups were significant, data were analysed using GraphPad Prism version 6.0 for Windows (GraphPad Software). The non-parametric Mann-Whitney test was used to estimate the relations between the two groups followed by the Benjamini and Hochberg multiple correction procedure. Statistical significances were accepted at $P<0.05$.

5.4 RESULTS

5.4.1 Survival and transit of *B. longum* DPC 6315 in BALB/c mice

Quantification of the numbers of the administered *B. longum* DPC 6315 strain in the faeces of mice confirmed its gastrointestinal transit and survival. Stool recovery of *B. longum* DPC 6315 was ~4.4×10^4 CFU/g faeces after 6 weeks of feeding the administered strain.

5.4.2 Effect of dietary supplementation with *B. longum* DPC 6315 on host tissue fatty acid composition

To investigate the effects of dietary supplementation with *B. longum* DPC 6315 on fatty acid composition of host tissues, fatty acid profiling was performed on liver and brain tissue.

In mice supplemented with *B. longum* DPC 6315 and the unsupplemented group, no significant difference in fatty acid concentration between the two groups in the liver was observed for the following fatty acids (Table 5.1): palmitic acid (16:0), palmitoleic acid (16:1 c9), oleic acid (18:1 c9), linolenic acid (18:3n-3), *c*9, *t*11 CLA, dihomo-γ-linolenic acid (20:3n-6), arachidonic acid (20:4n-6), DPA (22:5n-3) and DHA (Table 5.1).

In the brain, no significant difference in fatty acid concentration between the two groups was observed for the following fatty acids (Table 5.2): palmitic acid, palmitoleic acid, oleic acid, linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, EPA, DPA and DHA (Table 5.2).

Some significant differences in the fatty acid composition were observed between the two mouse groups, as mice that received *B. longum* DPC 6315 had significantly lower ($P<0.01$) levels of linoleic acid in the liver, (14.0 ± 0.3 g/100g FAME) compared to the unsupplemented group (15.4 ± 0.4 g/100g FAME) (Table 5.1), significantly lower ($P<0.001$) levels of γ-linolenic acid in the liver (0.1 ± 0.01 g/100g FAME) compared to the unsupplemented group (0.2 ± 0.01 g/100g FAME) (Table 5.1) and significantly lower ($P<0.05$) levels of stearic acid in the brain (22.3 ± 0.4 g/100g FAME) compared to the unsupplemented group (23.2 ± 0.2) (Table 5.2). In addition, mice that received *B. longum* DPC 6315 had significantly higher ($P<0.05$) concentrations of stearic acid (8.1 ± 0.2 g/100g FAME) compared to the unsupplemented group (7.4 ± 0.4 g/100g FAME), EPA (1.2 ± 0.01 g/100g FAME) compared to unsupplemented group 0.9 ± 0.1 g/100g FAME), and DPA (0.3 ± 0.1) compared to the unsupplemented group (0.2 ± 0.02 g/100g FAME) in the liver (Table 5.1).

5.4.3 Microbial compositional analysis by pyrosequencing

After removing low-quality 16S rRNA sequences, 425,285 from a total of 509,878 (83.41%) sequences passed the quality check and were used for further analysis. The number of reads per sample is shown in Table 5.3. In total 424,724 (99.86%) sequences were classified at the phylum level, 373,513 (73.25%) at the family level and 176, 228 (34.56%) at the genus level. Species-richness, coverage, and diversity estimations were calculated for each group (Table 5.3).

5.4.4 Alpha-diversity based analyses

The rarefraction curves and alpha diversities were calculated for each group. Chao1, Simpson and the Shannon diversity indices for the OTU's are shown in Table 5.3. The Shannon indices for the test group fed α-linolenic acid supplemented with *B. longum* DPC 6315 ranged from 6.81 to 7.82, while those for the group fed α-linolenic acid only ranged from 6.49 to 7.47. At the 97% similarity level, the Shannon index (a metric for community diversity) showed a high level of overall biodiversity within all samples with values exceeding 6.4 (Table 5.3).

Chao1 richness also indicated a sufficient level of overall diversity (Table 5.3). Rarefaction curves for each data set were parallel or approaching parallel with the *x* axis, which indicated that the total bacterial diversity present within these was well represented and that additional sampling would yield only a limited increase in species richness (Fig. 5.1). Sample 9 (fed high fat diet supplemented with *B. longum* DPC 6315) possibly possesses a considerably larger diversity than the other mouse caecal samples based on its Chao1 value (5230.9) (Table 5.3: Fig. 5.1) and observed species value (2645) (Table 5.3). This sample could also possess a large number of OTU's with single reads which would be recorded as single species which would lead to the sample's high Chao1 and observed species values.

5.4.5 Beta-diversity based analyses

Beta-diversity of the two mouse groups were estimated using the phylogenetic based unweighted and weighted UniFrac distance metrics and the non-phylogenetic Bray-Curtis dissimilarity distance metric. PCoA plots generated using Bray Curtis and both

UniFrac metrics, indicated that samples from the two mouse groups did not appear to cluster into distinct groups. A cladogram generated using UPGMA clustering of the 19 mouse microbiota datasets showed no obvious clustering of the mouse samples. This held true for UPGMA clustering using all metrics.

5.4.6 Analysis of the microbiota of mouse samples

1. Phylum level

The caecal contents of mice from the group fed a high fat diet supplemented with *B. longum* DPC 6315 contained significantly (p=0.0076) higher (0.2 fold increase) proportions of *Firmicutes*, 72% of the relative proportion (range 67.8%-77.6%) (Fig. 5.3 (a)), compared to the unsupplemented group, 61% of the relative proportion (range 48%-71.8%) (Fig. 5.3 (b)). The next dominant group was *Bacteriodetes* which were present at significantly (*P*=0.0057) higher (0.7 fold increase) proportions in the unsupplemented group (29.1%, range 17.8%-47.2%), than in the group fed *B. longum* DPC 6315 (17.2%, range 10.3%-23.5%). The microbiota of the two mouse groups also contained sub-dominant levels of *Deferribacteres* (Supplemented group: 10%, range 5%-15.8%), (Unsupplemented group: 9%, range 3.8%-12.6%). The phylum *Proteobacteria* was detected at 1% of the relative proportion in both groups (Supplemented group: range 0.3%-2.7%), (Unsupplemented group: range 0.4%-4%).

Two phyla in both groups were detected below 1% of the relative proportion - these were *Actinobacteria* (Supplemented group: 0.4%, range 0%-3.6%), (Unsupplemented group: 0.08%, range 0%-2.9%) and *Tenericutes* (Supplemented group: 0.04%, range 0%-0.1%) (Fig. 5.3 (a)), (Unsupplemented group: 0.03%, range 0%-0.07%)

(Fig. 5.3 (b)). The phylum *Ascomycota* was only detected in one of the mouse samples in the group supplemented with *B. longum* DPC 6315 and in two of those in the unsupplemented group. The phylum Candidate division TM7 was detected in three of the mouse samples in the group fed *B. longum* DPC 6315 and in two of those in the unsupplemented group.

2. Family level

At the family level, *Lachnospiraceae* were the dominant family in the two mouse groups making up 51% (range 31.54%-50.7%), (Fig. 5.4 (a)) in the group supplemented with *B. longum* DPC 6315 and 45% (range 27.4%-56.2%), (Fig. 5.4 (b)) in the unsupplemented group. This family was followed in abundance by *Rikenellaceae* (Supplemented group: 11.2%, range 5.18%-15.2%), (Unsupplemented group: 18.8%, range 11.67%-30.12%), *Ruminococcaceae* (Supplemented group: 15.5%, range 11.65%-20.97%), (Unsupplemented group: 12.89%, range 8.7%-18.93%), *Deferribacteraceae* (Supplemented group: 9.7%, range 5.1%-15.77%), (Unsupplemented group: 8.5%, range 3.84%-12.64%), *Bacteriodaceae* (Supplemented group: 1.74%, range 1.22%-3.77%), (Unsupplemented group: 3.56%, range 1.68%-6.24%), *Porphyromonadaceae* (Supplemented group: 0.77%, range 0.42%-1.13%), (Unsupplemented group: 1.36%, range 0.55%-2.58%).

All the other families detected were below 1% of the total relative abundance in each group which included *Peptococcaceae* (Supplemented group: 0.87%, range 0.55%-1.27%), (Fig. 5.4 (a)) (Unsupplemented group: 0.79%, range 0.42%-1.45%), (Fig. 5.4 (b)), *Peptococcaceae (Clostridiales)* (Supplemented group: 0.49%, range 0.22%-1.07%),

(Unsupplemented group: 0.7%, range 0.29%-1.27%), *Desulfovibrionaceae* (Supplemented group: 0.52%, range 0.15%-0.8%), (Unsupplemented group: 0.46%, range 0.13%-0.92%) and *Ersipelotrichaceae* (Supplemented group: 0.61%, range 0.07%-2.08%), (Unsupplemented group: 0.22%, range 0.04%-0.63%).

All the other families detected were below 0.5% of the total relative abundance in both groups *Moraxellaceae* (Supplemented group: 0.11%, range 0%-1.02%), (Fig. 5.4 (a)), (Unsupplemented group: 0.37%, range 0%-2.18%), (Fig. 5.4 (b), *Lactobacillaceae* (Supplemented group: 0.36%, range 0%-1.82%), (Unsupplemented group: 0.06%, range 0%-0.31%), *Bifidobactericaeae* (Supplemented group: 0.35%, range 0%-3.55%), (Unsupplemented group: 0.03%, range 0%-0.16%), *Clostridiaceae* (Supplemented group: 0.26%, range 0%-0.77%), (Unsupplemented group: 0.08%, range 0%-0.22%), (Unsupplemented group: 0.12%, range 0.02%-0.38%), *Streptococcaceae* (Supplemented group: 0.13%, range 0.03%-0.3%), (Unsupplemented group: 0.12%, range 0.02%-0.38%), *Rhodospirillaceae* (Supplemented group: 0.09%, range 0%-0.29%), (Unsupplemented group: 0.1%, range 0%-0.24%), *Enterobactericeae* (Supplemented group: 0.1%, range 0%-0.58%), (Unsupplemented group: 0.06%, range 0%-0.4%), and *Leuconostocaceae* (Supplemented group: 0.008%, range 0%-0.04%), (Unsupplemented group: 0.007%, range 0%-0.044%).

3. Genus level

The most abundant genus in both mouse groups was *Alistipes* which was detected at a significantly (*P*=0.04) higher (0.8 fold increase) abundance in the group fed the high fat diet only (15.84%, range 9.9%-24.8%), (Fig. 5.5 (b)) compared to 9%, (range 4.95%-

11.79%) in the group fed the high fat diet supplemented with *B. longum* DPC 6315 (Fig. 5 (a)). This genus was followed in abundance by *Mucispirillum* (Supplemented group: 9.7%, range 5%-15.8%), (Unsupplemented group: 8.5%, range 3.84%-12.64%), *Ruminococcaceae Incertae Sedis* (Supplemented group: 6.84%, range 5%-8.8%), (Unsupplemented group: 5.8%, range 3.88%-8.3%), *Oscillibacter* (Supplemented group: 2.75%, range 1.3%-5.85%), (Unsupplemented group: 1.8%, range 1.14%-3.24%), *Bacteroides* (Supplemented group: 1.7%, range 0.96%-3.8%), (Unsupplemented group: 3.6%, range 1.4%-6.2%), *Anaerotruncus* (Supplemented group: 2.3%, range 1.8%-3.18%), (Unsupplemented group: 2%, range 1.25%-2.96%), and *Lachnospiraceae Incertae Sedis* (Supplemented group: 1.9%, range 1.23%-2.17%), (Unsupplemented group: 1.6%, range 0.87%-2.2%) and *Parabacteroides* (Supplemented group: 0.48%, range 0.8%-0.33%), (Fig. 5 (A), (Unsupplemented group: 0.8%, range 0.3%-2.2%) (Fig. 5.5 (b)).

Other genera detected below 1% of the total relative abundance in both groups included *Peptococcus* (Supplemented group: 0.7%, range 0.39%-1.11%), (Unsupplemented group: 0.6%, range 0.33%-1.3%), *Bilophila* (Supplemented group: 0.5%, range 0.15%-0.8%), (Unsupplemented group: 0.5%, range 0.13%-0.92%), *Coprococcus* (Supplemented group: 0.5%, range 0.23%-0.87%), (Unsupplemented group: 0.4%, range 0.17%-0.7%), *Odoribacter* (Supplemented group: 0.3%, range 0.07%-0.8%), (Unsupplemented group: 0.56%, range 0%-2.2%) and *Allobaculum* (Supplemented group: 0.58%, range 0.06%-2.06%), (Unsupplemented group: 0.21%, range 0.04%-0.63%). The genus *Roseburia* was detected at a significantly (P=0.041)

higher abundance (0.5 fold increase) in the supplemented group (0.5%, range 0.14%-1.05%) compared to the unsupplemented group (0.16%, range 0.04%-0.63%).

All the other genera were detected below 0.5% of the total relative abundance which included *Peptococcaceae Incertae Sedis* (Supplemented group: 0.17%, range 0.08%-0.44%), (Fig. 5.5 (a)), (Unsupplemented group: 0.37%, range 0%-0.9%), (Fig. 5.5 (b)) *Psychrobacter* (Supplemented group: 0.1%, range 0%-1%), (Unsupplemented group: 0.36%, range 0%-2.17%), *Lactobacillus* (Supplemented group: 0.36%, range 0%-1.8%), (Unsupplemented group: 0.06%, range 0%-0.31%), *Bifidobacterium* (Supplemented group: 0.35%, range 0%-3.5%), (Unsupplemented group: 0.03%, range 0%-0.16%), *Clostridium (Clostridiceae)* (Supplemented group: 0.24%, range 0%-0.76%), (Unsupplemented group: 0.07%, range 0%-0.21%), *Lactococcus* (Supplemented group: 0.1%, range 0.03%-0.2%), (Unsupplemented group: 0.1%, range 0.02%-0.37%), *Thalassospira* (Supplemented group: 0.09%, range 0%-0.29%), (Unsupplemented group: 0.1%, range 0%-0.24%), *Catabacter* (Supplemented group: 0.09%, range 0.04%-0.14%), (Unsupplemented group: 0.05%, range 0%-0.1%), and *Escherichia-Shigella* (Supplemented group: 0.09%, range 0%-0.5%), (Fig. 5.5 (a)), (Unsupplemented group: 0.05%, range 0%-0.35%) (Fig. 5.5 (b)).

5.5 DISCUSSION

As *B. longum* DPC 6315 has been identified as an EPS producer (Chapter 2) and potentially a bacteriocin producer (Chapter 2 and Chapter 3), any effects that the strain might have on the overall microbiota could be due to one or a combination of these potential probiotic traits. Quantification of the numbers of bacteria of the *B. longum* DPC 6315 strain recorded in the faeces of individual mice confirmed gastrointestinal transit and survival of *B. longum* DPC 6315. Faecal recovery was approximately was ~4.4×10^4 CFU/g faeces in mice that received the strain in combination with the high fat diet.

Fatty acid composition analysis revealed that mice that received *B. longum* DPC 6315 and the high fat diet had significantly lower levels of linoleic acid and γ-linolenic acid in the liver compared to unsupplemented mice. The significantly lower levels of both linoleic acid and γ-linolenic acid (a metabolite of linoleic acid) in the liver suggest that linoleic acid has been metabolised to some degree in this tissue. This metabolism could be due to the presence of *B. longum* DPC 6315 which has been found possess the capability to metabolise linoleic acid to form another linoleic acid conjugate CLA (Barrett *et al.*, 2007). Another possible explanation for this linoleic acid metabolism could be due to the presence of members of the genus *Roseburia*, which also have been reported to be able to convert linoleic acid to some of its precursors of CLA using the same biohydration pathway as that used by rumen bacteria (Devillard *et al.*, 2007). Indeed, some *Roseburia* species have been identified as being among the most active producers of CLA and CLNA in the GIT (Devillard *et al.*, 2007). Pyrosequencing revealed that in the supplemented mouse group there was a significant higher proportion of the genus *Roseburia* compared to the unsupplemented group. While both *B. longum*

DPC 6315 and some members of the genus *Roseburia* are capable of converting linoleic acid to *c*9, *t*11 CLA, no significant difference in *c*9, *t*11 CLA concentrations were recorded in the liver tissues taken from mice from the supplemented and unsupplemented groups in this study, and in the brain tissues taken from mice from both the groups no *c*9, *t*11 CLA was detected. A possible reason for both of these observations could be that the diet fed to the mice was high in α-linolenic acid instead of linoleic acid from which *c*9, *t*11 CLA is produced. Possibly, levels of linoleic acid in the high fat diet fed in this study were too low to see any significant differences in *c*9, *t*11 CLA content in the liver and brain tissues examined for fatty acid composition. It appears in this study that in the supplemented group, linolenic acid has been metabolised to form EPA which was then converted to DPA with significantly higher levels of both fatty acids found in the livers of mice in this group. It appears possible that the metabolic activities of *B. longum* DPC 6315 in this study are more focused on producing metabolites of α-linolenic acid such as EPA (Wall *et al.*, 2010), and then in turn DPA (Burdge *et al.*, 2002) (as observed in the significantly higher levels of both fatty acids in the liver) instead of *c*9, *t*11 CLA (a metabolite of linoleic acid).

The presence of significantly lower stearic acid levels in the brain of mice fed *B. longum* DPC 6315 compared to the unsupplemented mouse group suggests that linoleic acid is not being metabolised by *B. longum* DPC 6315 in this tissue, as stearic acid is a metabolite of linoleic acid (Devillard *et al.*, 2007). No *c*9, *t*11 CLA was recorded in the brain tissues of mice from both the supplemented and the unsupplemented group. This suggests in this study, that in the presence of a diet rich in α-linolenic acid, *B. longum* DPC 6315 does not produce *c*9, *t*11 CLA in this tissue *in vivo*. In contrast the presence of

significantly higher levels of stearic acid in the liver of mice supplemented with *B. longum* DPC 6315 suggest that linoleic acid is being metabolised to some degree in this tissue, although no significant difference in *c*9, *t*11 CLA was recorded between the supplemented and unsupplemented groups in this study. As discussed above, a possible reason for this was the diet fed to the mice was high in α-linolenic acid instead of linoleic acid. So as discussed above, it appears in this study that in the supplemented group, linolenic acid has been metabolised by *B. longum* DPC 6315 to form EPA, and in turn DPA as observed by the significantly higher levels of both fatty acids found in the liver of mice in this group.

Eicosanoids derived from arachidonic acid are regarded as being pro-inflammatory in nature (Bagga *et al.*, 2003; Calder, 2008), whereas eicosanoids derived from EPA are reported to be less inflammatory or even anti-inflammatory in nature (Bagga *et al.*, 2003; Calder, 2008; Robinson & Stone, 2006). EPA can be further metabolised to DHA, which is one of the main polyunsaturated fatty acids found in the brain (Wall *et al.*, 2010). It has a vital role in fetal brain development and is critical in the newborn for proper development and intelligence (Salem *et al.*, 1996). It has been demonstrated in some studies that DHA provides support to learning and memory events in animal models of Alzheimer's disease (Hashimoto *et al.*, 2002) and brain injury (Wu *et al.*, 2004). Similar findings were recorded in the study by Wall et al. (2010), where *B. breve* NCIMB 702258 and the precursor for EPA and DHA, α-linolenic acid was fed to mice, the control group received α-linolenic acid only. Significantly ($P<0.05$) higher levels of EPA in the liver and DHA in the brain compared to the control group were reported.

DPA has been reported in some *ex vivo* studies on rabbit and human blood platelets (Akiba *et al.*, 2000; Phang *et al.*, 2009) to inhibit platelet aggregation which is an early event in the development of thrombosis. DPA may also possess positive effects on the control of wound healing *in vitro* (Kanayasu-Toyoda *et al.*, 1996). Endothelial cell migration and proliferation are important processes in the control of wound-healing response of blood vessels. Direct pretreatment of endothelial cells with DPA (0.01-1 mg/ml) resulted in a dose-dependent increase in migration. Maximum stimulation of endothelial cell migration was achieved at a concentration one-tenth (0.5 mg/ml) of that required for maximal stimulation by EPA pretreatment (5 mg/ml) indicating that DPA is a potent stimulator of endothelial cell migration (Kanayasu-Toyoda *et al.*, 1996).

In agreement with previous work (Ley *et al*., 2005; Ley *et al*., 2006; Turnbaugh *et al*., 2006; Wall *et al*., 2012), taxonomy-based analysis showed that, at the phylum level, the mouse caecal microbiota was dominated by *Firmicutes* and *Bacteroidetes* (together harboring on average 90% of sequences). Whereas it is acknowledged that pyrosequencing of the 16S rRNA genes, as performed in this study, did not provide quantitative population data, it did yield an overview of the effects of administration of *B. longum* DPC 6315 on the entire mouse microbial population. *Firmicutes* were the most dominant phyla in both mouse groups, and were found at a significantly higher relative abundance in the mice fed α-linolenic acid supplemented with *B. longum* DPC 6315 compared to those fed α-linolenic acid only. The study performed by Turnbaugh et al. (2006) demonstrated that microbiota transplanted from obese mice had a significantly ($P<0.05$) higher relative abundance of *Firmicutes* compared with lean mice microbiota. Other studies performed in both mice and humans (Ley *et al.*, 2005; Ley *et al.*, 2006)

have shown a significantly greater relative abundance of *Firmicutes* in obese subjects compared to lean controls. These studies also showed a significant relative reduction in *Bacteroidetes* in obese animals compared to lean ones. In this study *Bacteroidetes* were detected at a significantly higher abundance in the mice fed the high fat diet only. There were no significant differences in bodyweight or fat composition between the two groups of mice so this would suggest that *B. longum* DPC 6315 has no effect on the energy harvesting capabilities of the overall mouse microbiota.

At the family level *Lachnospiraceae* were the most abundant in each mouse group with a higher relative abundance in the supplemented group. This is in contrast to results reported by Wall et al. (2012) which found that feeding both *B. breve* DPC 6330 and *B. breve* NCIMB 702258 caused a significantly ($P<0.05$) lower proportion of *Lachnospiraceae* than did no supplementation. *Rikenellaceae, Porphyromonadaceae* and *Bacteriodaceae* were more abundant in the unsupplemented mouse group, while *Bifidobacteriaceae* and *Lactobacillaceae* were more abundant in the group supplemented with *B. longum* DPC 6315. *Alistipes* was the most abundant genus detected in both mouse groups being significantly higher in the unsupplemented group. This genus is closely related to the genus *Bacteroides* and contains species such as *Alistipes finegoldii* which has been found to cause bacteremia in colon cancer patients who had undergone surgical resection (Fenner *et al.*, 2007). As stated earlier the relative abundance of the genus *Roseburia* was significantly higher in the group supplemented with *B. longum* DPC 6315. This genus has been reported to contain species that produce butyrate as a fermentation end product. Butyrate has been shown to be a major source of energy for colonic epithelial cells (Cummings, 1981; Cummings *et al*., 1987; Roediger *et al*., 1982). It has

been found to provide the body with 7-10% of its energy needs (Roy *et al*., 2006). Butyrate has also been shown to have anti-carcinogenic effects by preventing mutagens at their active site, molecular or DNA level (Smith, 1995). It is possible that the supplementation of *B. longum* DPC 6315 is leading to a higher relative abundance of this genus which contains butyrate producers.

An interesting observation was that no significant difference in the relative abundance of the genus *Bifidobacterium* was recorded between the two mouse groups, even though the supplemented group received ~1×10^9 CFU/day of *B. longum* DPC 6315. This was also seen in the study Wall et al. (2012) in which no significant difference in the relative abundance of the genus *Bifidobacterium* was observed between a mouse group fed ~1×10^9 CFU/day *B. breve* NCIMB 702258, a group fed ~1×10^9 CFU/day *B. breve* DPC 6330 and an unsupplemented group (fed no *Bifidobacterium* strain). A possible explanation for this is that the presence of the *Bifidobacterium* strain being fed to the mice is inhibiting the growth of the *Bifidobacterium* species that are already present in the mouse caecum. *B. longum* DPC 6315 could possibly be producing a lantibiotic *in vivo*, as in Chapter 2, *B. longum* DPC 6315 was found to contain the lantibiotic prepeptide gene *lanA*, and in Chapter 3 to possess a lantibiotic operon with ~99% sequence identity to one found in *B. longum* DJ010A (Lee *et al.*, 2011) which had been found to inhibit some bifidobacterial strains such as those from the species *longum*, *adolescentis*, *breve*, *bifidum* and *animalis*. In this study if *B. longum* DPC 6315 were producing a lantibiotic *in vivo* it could have inhibitory effects on other bifidobacterial strains present in the mouse caecum. The *Bifidobacterium* strain being fed at very high numbers (~10^9 CFU) daily to the mice (such as *B. longum* DPC 6315) could therefore be

out-competing the other *Bifidobacterium* species already present in the mouse caecum. This would possibly lead to lower numbers of the *Bifidobacterium* species naturally present in the mouse caecum being recorded in the supplemented group of mice, with most of the recorded *Bifidobacterium* species in the supplemented group being those fed on a daily basis to the mice, and those in the unsupplemented group being those *Bifidobacterium* species that are already present in the mouse caecum.

In conclusion, supplementation with *B. longum* DPC 6315 led to significant differences in fatty acid composition in both the murine liver and brain tissues compared to supplementation with α-linolenic acid only. *B. longum* DPC 6315 supplementation also led to significant differences in the overall caecal microbiota at both the phylum and genus level.

5.6 ACKNOWLEDGEMENT

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Table 5.1 Fatty acid profile in the liver of mice fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315 or an unsupplemented diet for 6 weeks[1].

Fatty acid	**Mice fed *B. longum* DPC 6315**	**Unsupplemented mice**
	g/100g FAME	*g/100g FAME*
Palmitic acid, 16:0	26.1 ± 0.3	25.7 ± 0.5
Palmitoleic acid, 16:1c9	3.0 ± 0.2	2.7 ± 0.2
Stearic acid, 18:0	8.1 ± 0.2 [a]	7.4 ± 0.3 [b]
Oleic acid, 18:1c9	30.1 ± 0.7	31.7 ± 0.8
Linoleic acid, 18:2n-6	14.0 ± 0.3 [a]	15.4 ± 0.4 [b]
*c*9, *t*11 CLA	0.04 ± 0.2	0.03 ± 0.1
Linolenic acid, 18:3n-3	5.2 ± 0.2	5.1 ± 0.2
γ-Linolenic acid, 18:3n-6	0.1 ± 0.01 [a]	0.2 ± 0.01 [b]
Dihomo-γ-linolenic acid, 20:3n-6	0.5 ± 0.02	0.4 ± 0.03
Arachidonic acid, 20:4n-6	2.7 ± 0.1	2.8 ± 0.2
EPA, 20:5n-3	1.2 ± 0.1 [a]	0.9 ± 0.1 [b]
DPA, 22:5n-3	0.3 ± 0.1 [a]	0.2 ± 0.02 [b]
DHA, 22:6n-3	2.3 ± 0.2	2.2 ± 0.2

[1]All values are means ±SEMs; *n*=10 mice per group. Values in the same row with different superscript letters are significantly different, $P<0.05$ (Unpaired T-test). Fatty acid methyl ester (FAME).

Table 5.2 Fatty acid profile in the brain of mice fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315 or an unsupplemented diet for 6 weeks[1].

Fatty acid	**Mice fed *B. longum* DPC 6315**	**Unsupplemented mice**
	g/100g FAME	*g/100g FAME*
Palmitic acid, 16:0	30.9 ± 0.5	30.4 ± 0.7
Palmitoleic acid, 16:1c9	0.8 ± 0.02	0.7 ± 0.02
Stearic acid, 18:0	22.3 ± 0.4 [a]	23.2 ± 0.2 [b]
Oleic acid, 18:1c9	19.6 ± 0.3	20.01 ± 0.1
Linoleic acid, 18:2n-6	0.9 ± 0.02	0.9 ± 0.02
*c*9, *t*11 CLA	ND	ND
γ-Linolenic acid, 18:3n-6	0.3 ± 0.01	0.3 ± 0.02
Dihomo-γ-linolenic acid, 20:3n-6	0.4 ± 0.01	0.4 ± 0.2
Arachidonic acid, 20:4n-6	5.9 ± 0.1	5.9 ± 0.01
EPA, 20:5n-3	0.1 ± 0.04	0.1 ± 0.2
DPA, 22:5n-3	0.2 ± 0.01	0.2 ± 0.003
DHA, 22:6n-3	7.0 ± 0.4	7.2 ± 0.01

[1]All values are means ±SEMs; *n*=10 mice per group. Values in the same row with different superscript letters are significantly different, $P<0.05$ (Unpaired T-test). ND = Not detected.

Table 5.3 Alpha diversity measures for each mouse sample.

Sample	Chao1	Shannon	Simpson	PD whole tree	Observed species	Total number of Reads
1	1636.3	6.95	0.97	47.4	1120	20665
2	2500.3	7.28	0.98	57.68	1392	20624
3	1984.2	7.54	0.98	51.69	1248	18048
4	2343.3	6.81	0.96	54.86	1342	22225
5	1842.2	7.18	0.98	49.56	1207	24258
6	3281.8	7.82	0.98	70.19	1745	24822
7	2737	7.45	0.98	65.85	1636	28640
8	1808.1	7.07	0.97	48.73	1224	22809
9	5230.9	7.79	0.98	101.05	2645	29732
10	2270.9	7.3	0.98	57.65	1389	23463
11	1998.3	6.49	0.96	51.16	1080	20196
12	1672.6	6.5	0.96	42.68	1009	20692
13	1677.5	6.73	0.97	45.62	1040	23479
14	2802.7	6.89	0.96	62.72	1479	21943
15	1614.4	7.09	0.98	46.7	1108	23374
16	2316.8	7.06	0.98	54.94	1336	16192
17	1848.4	7.47	0.98	50.76	1189	21058
18	1557.7	6.76	0.97	45.7	1067	20491
19	2184.2	7.02	0.97	55.1	1264	22572

Legend: Samples 1-10 fed *B. longum* DPC 6315 and high fat diet. Samples 11-19 fed α-linolenic acid enriched diet only.

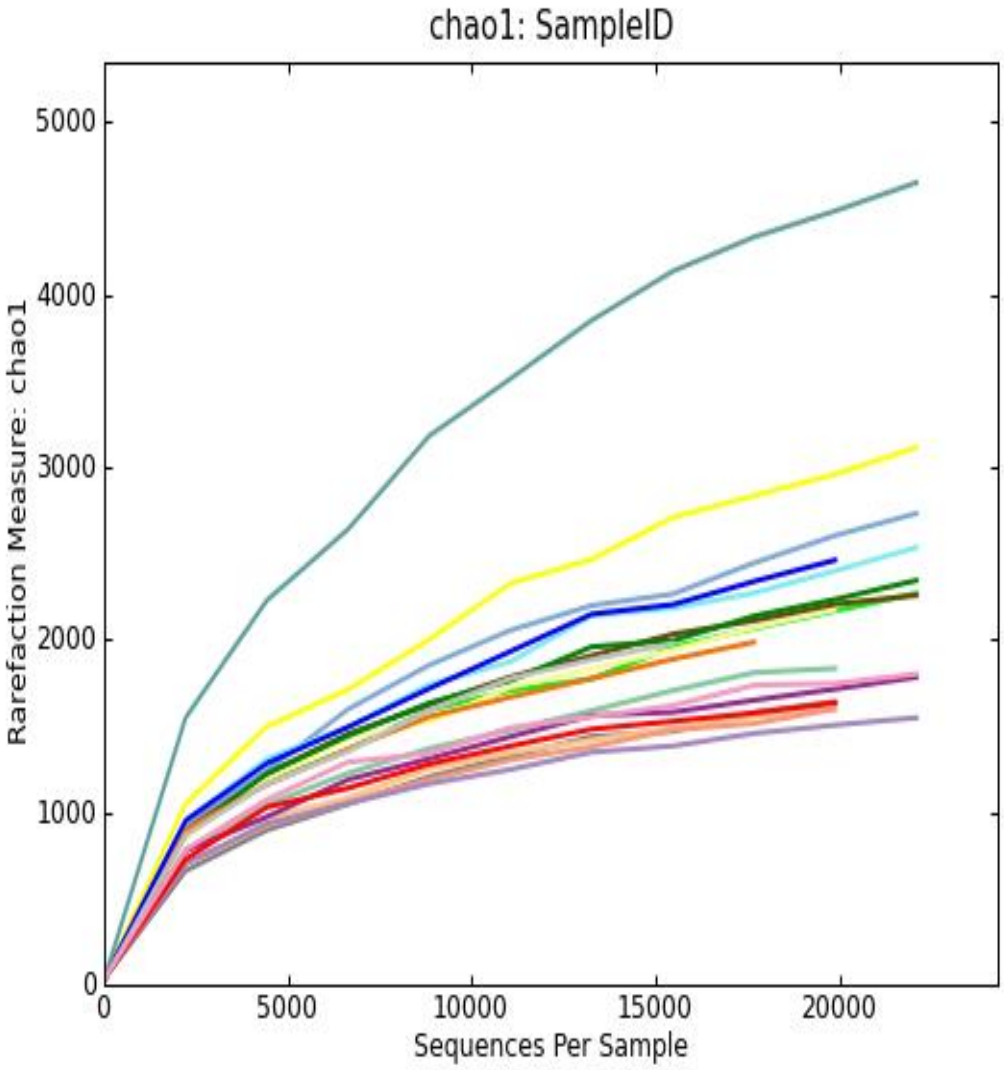

Figure 5.1 Rarefaction plot for assessment of OTU coverage. Each curve represents an individual mouse sample.

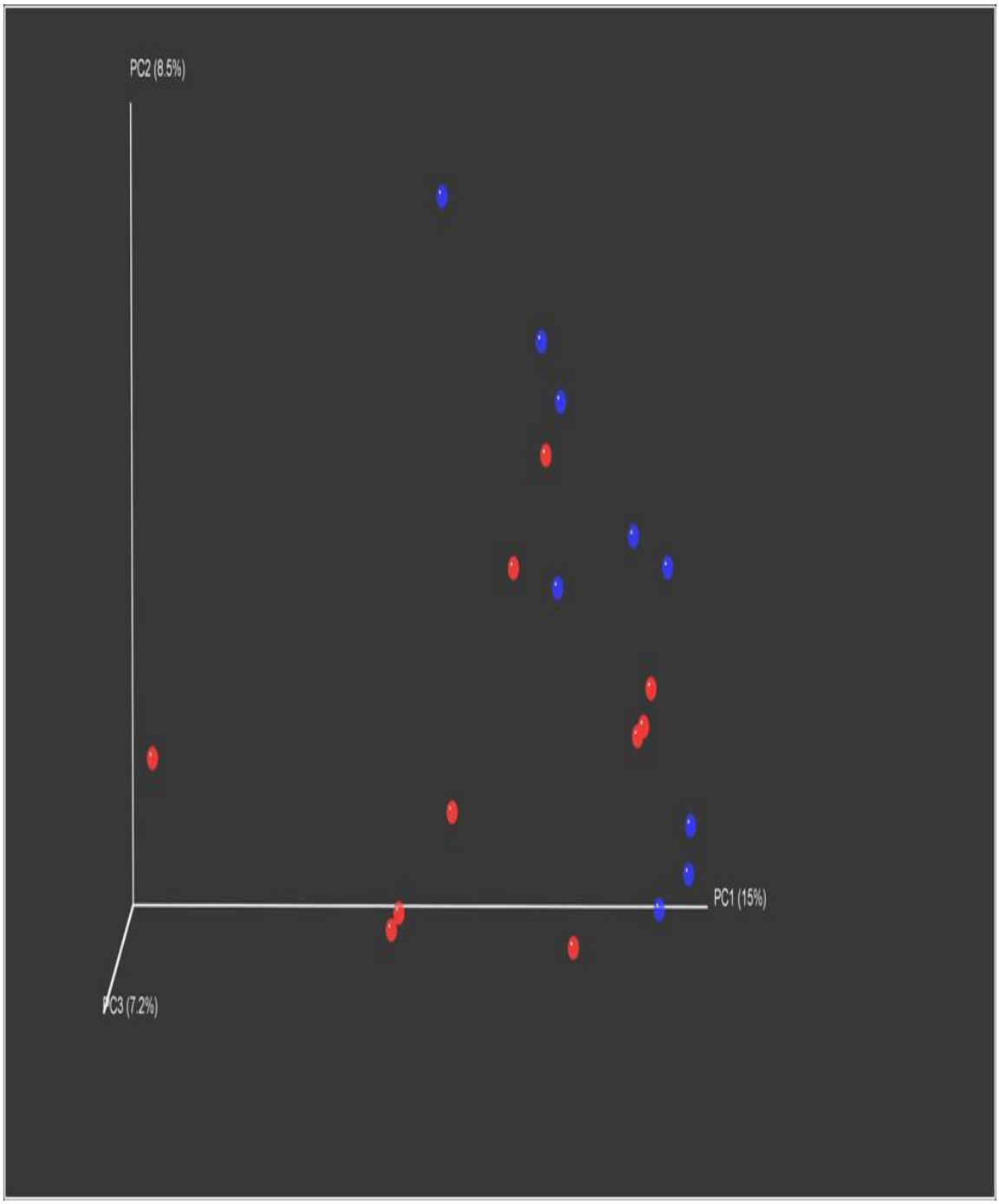

Figure 5.2 (a) PCoA of unweighted UniFrac distances of 16S rRNA sequences.

Legend: Red points = Samples 1-10 fed α-linolenic acid enriched diet and *B. longum* DPC 6315.
Blue points = Samples 11-19 fed α-linolenic acid enriched diet only.

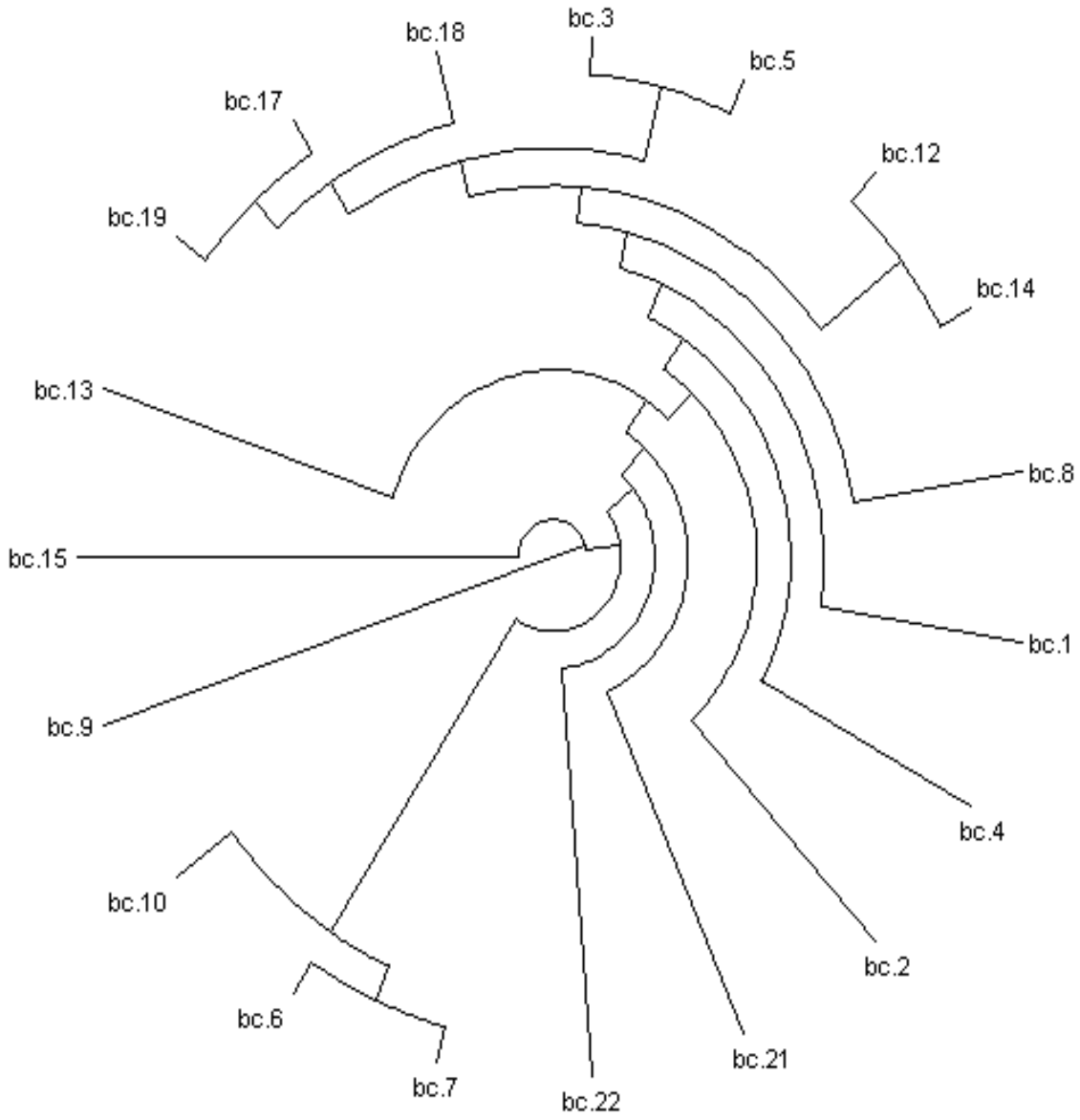

Figure 5.2 (b) A cladogram generated using unweighted pair group method with UPGMA clustering of the 19 mouse microbiota datasets.

Legend: bc.1-10 = Samples 1-10 fed α-linolenic acid enriched diet and *B. longum* DPC 6315.
bc.12-15, 17-19, 21 and 22 = Samples fed α-linolenic acid enriched diet only.

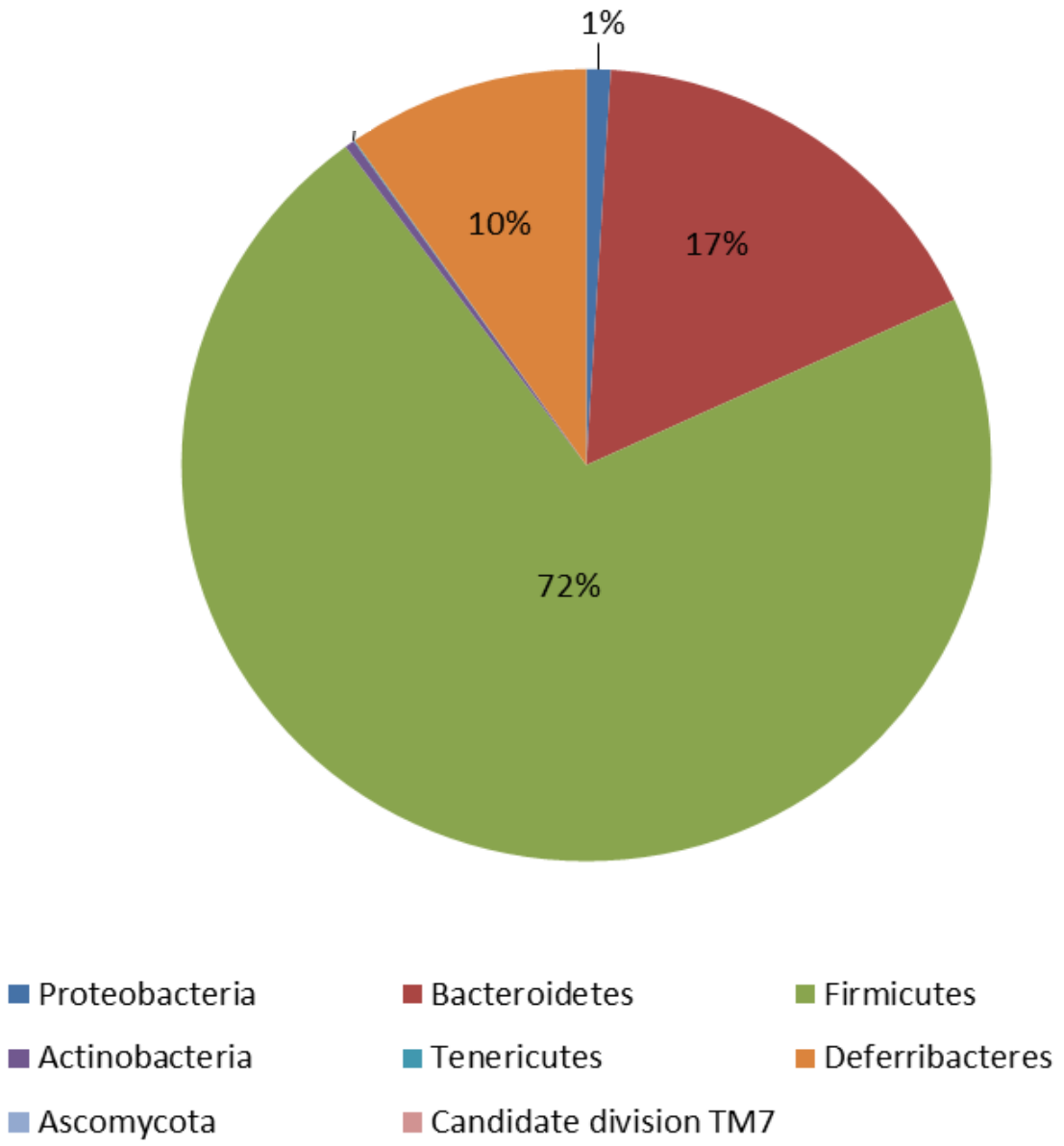

Figure 5.3 (a) The aggregate relative proportion of phyla of the caecal microbiota of the mouse group fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315.

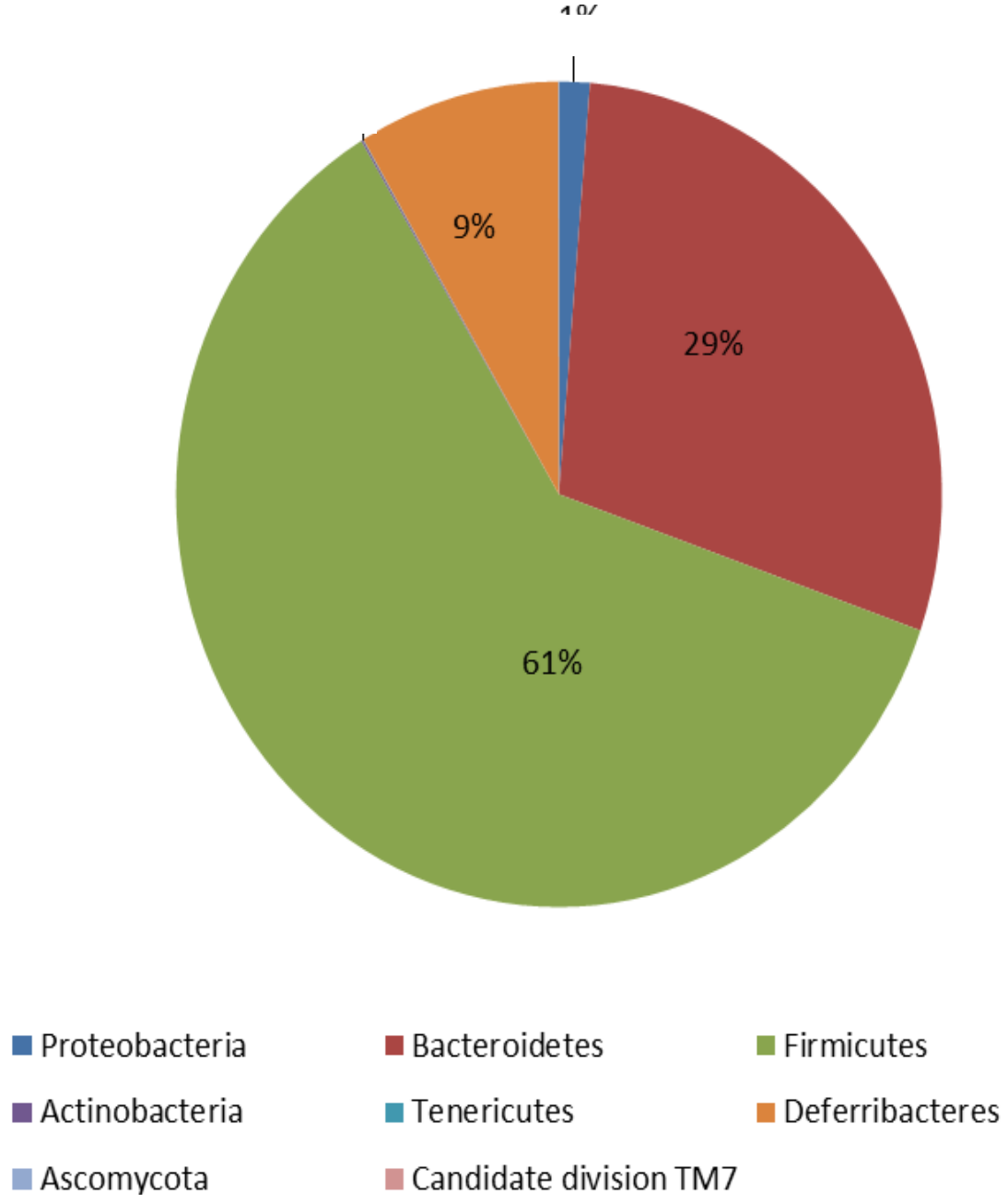

Figure 5.3 (b) The aggregate relative proportion of phyla of the caecal microbiota of the mouse group fed α-linolenic acid enriched diet only.

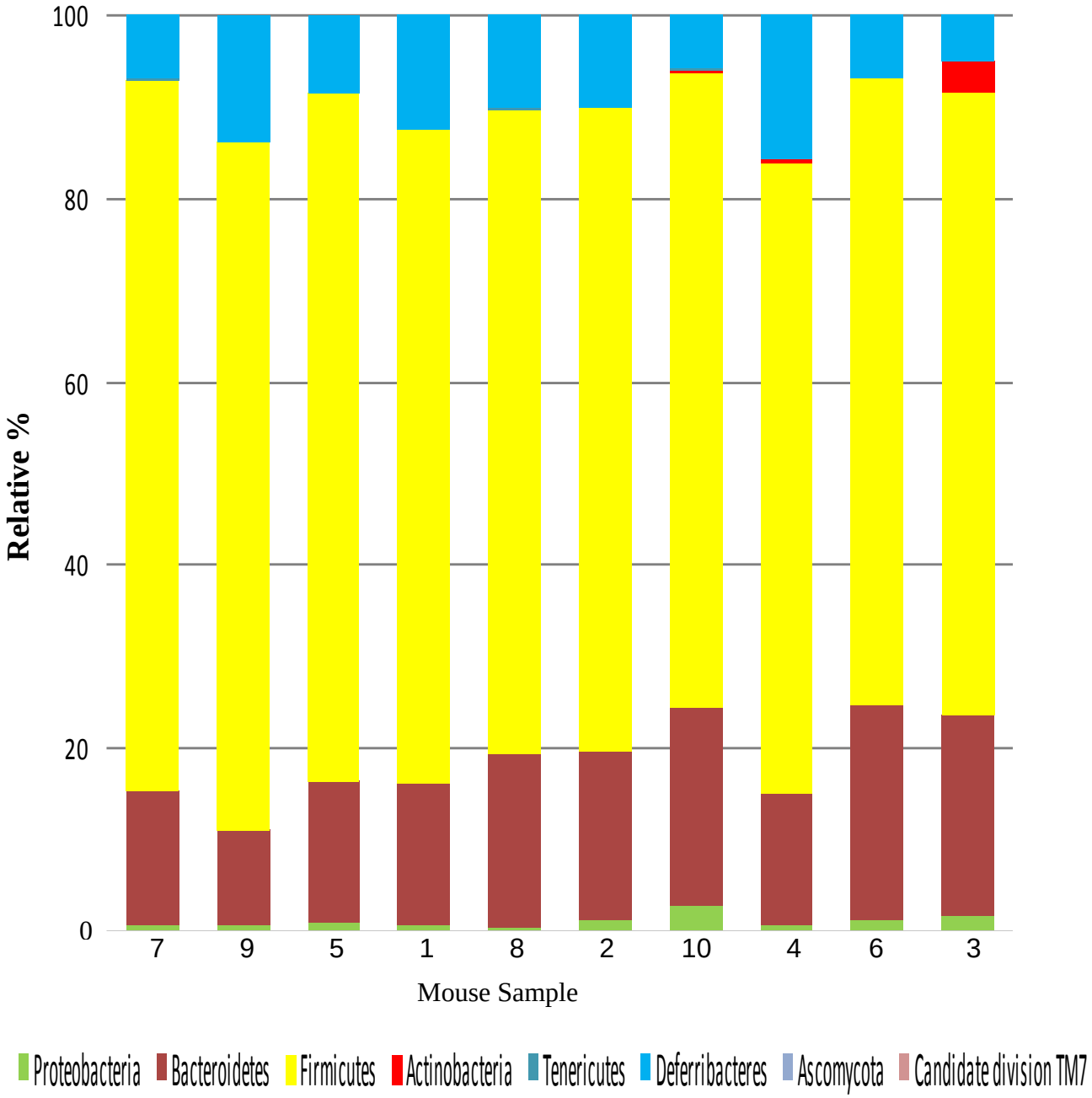

Figure 5.3 (c) Inter-individual variation in the proportion of the major phyla between the mouse samples fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315. Samples are ordered from left according to *Firmicutes* proportion.

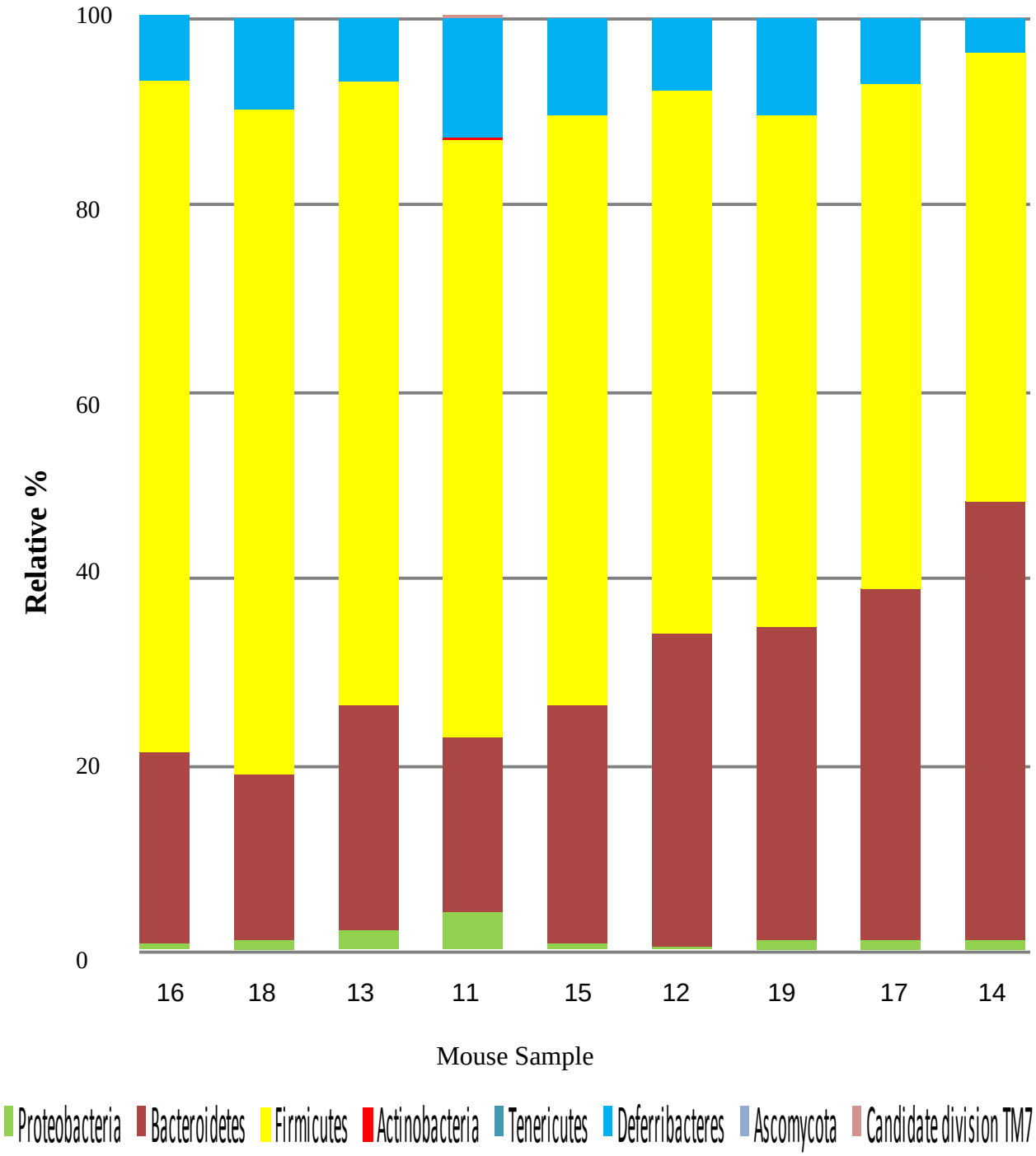

Figure 5.3 (d) Inter-individual variation in the proportion of the major phyla in the mouse samples fed α-linolenic acid enriched diet only.
Samples are ordered from left according to *Firmicutes* proportion.

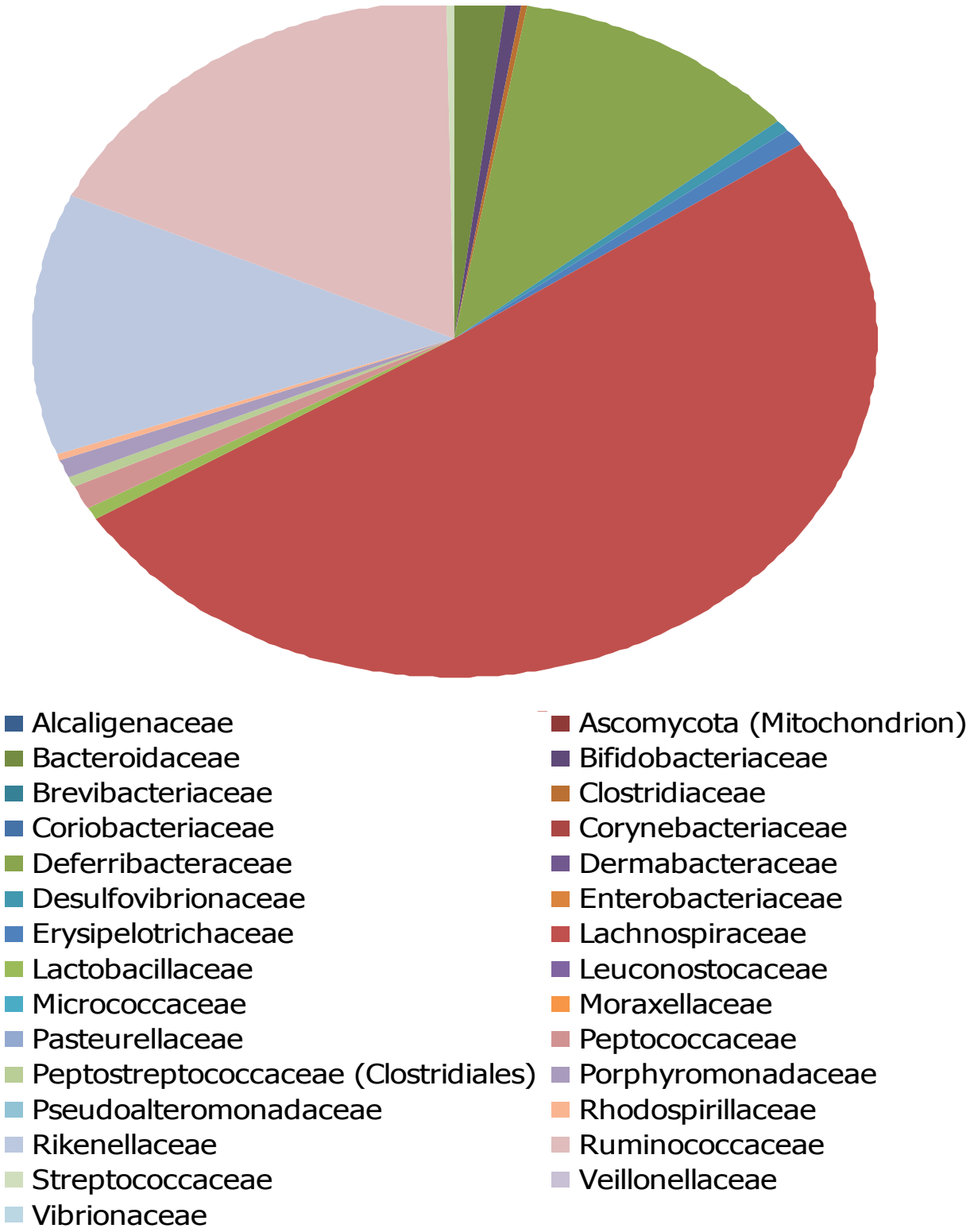

Figure 5.4 (a) The aggregate relative proportion of family-level caecal microbiota of the mouse group fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315.

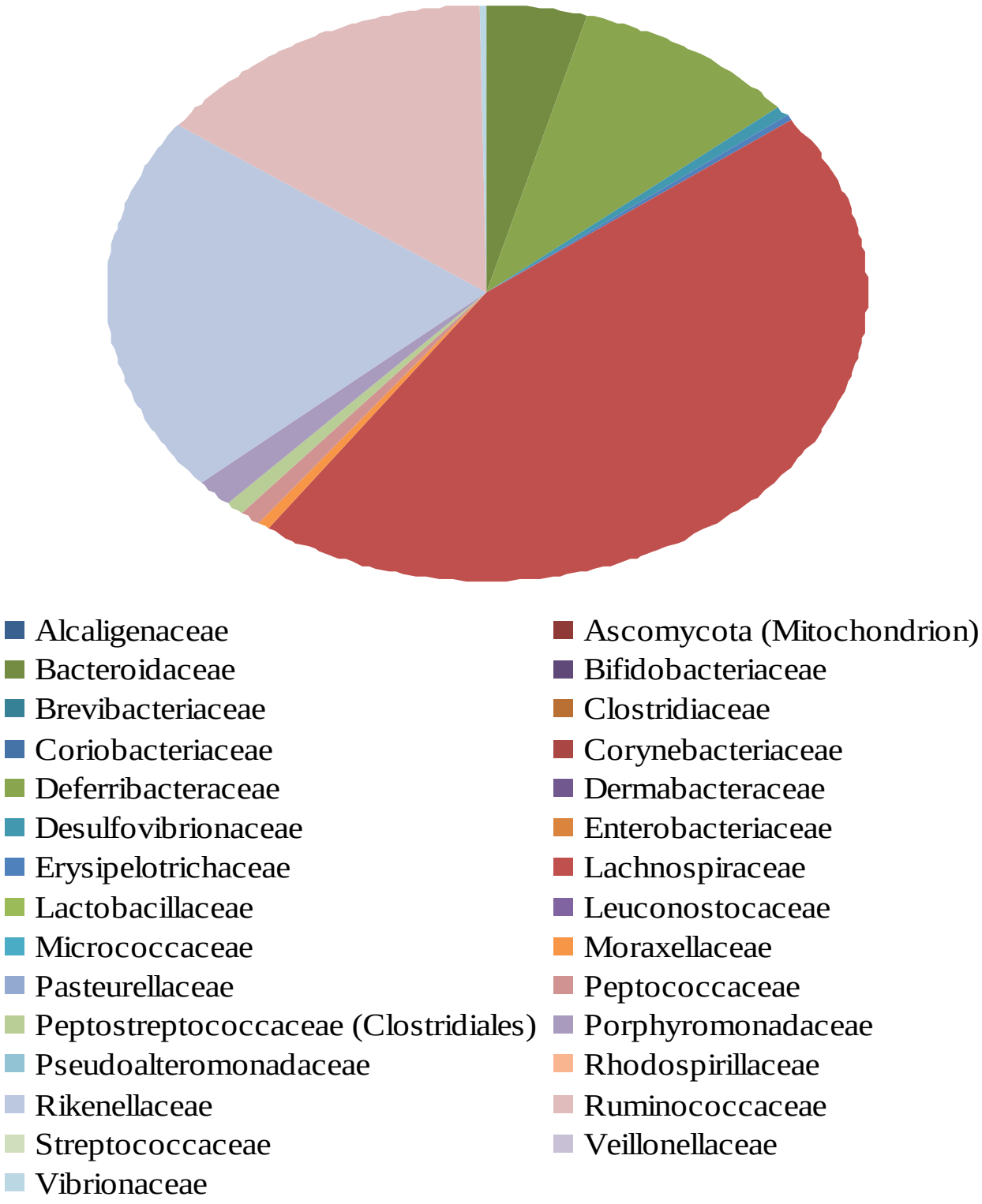

Figure 5.4 (b) The aggregate relative proportion of family-level caecal microbiota of the mouse group fed α-linolenic acid enriched diet only.

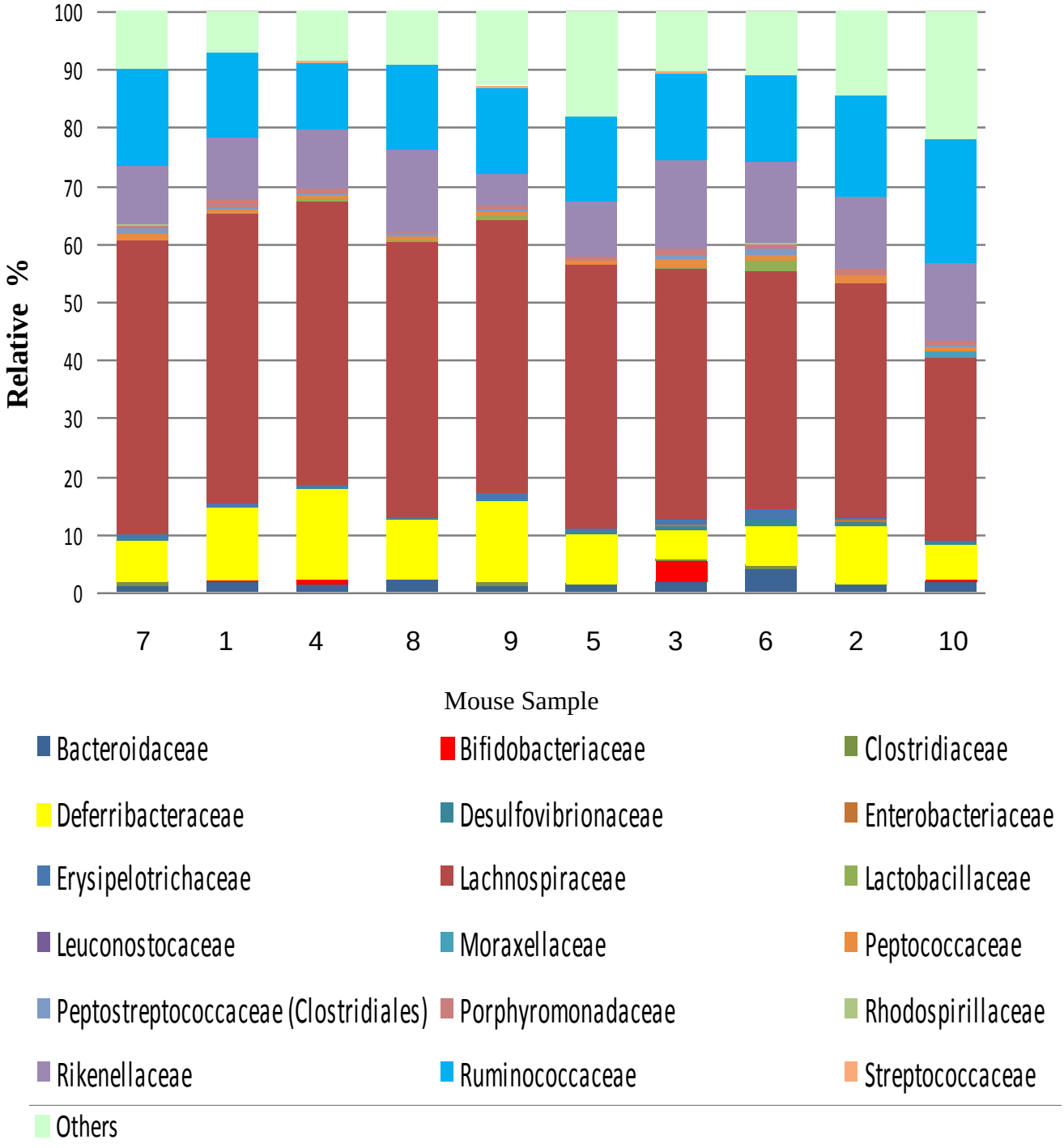

Figure 5.4 (c) Inter-individual variation in the proportion of the major family-level caecal microbiota of the mouse group fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315.

Samples are ordered from left according to *Firmicutes* proportion.

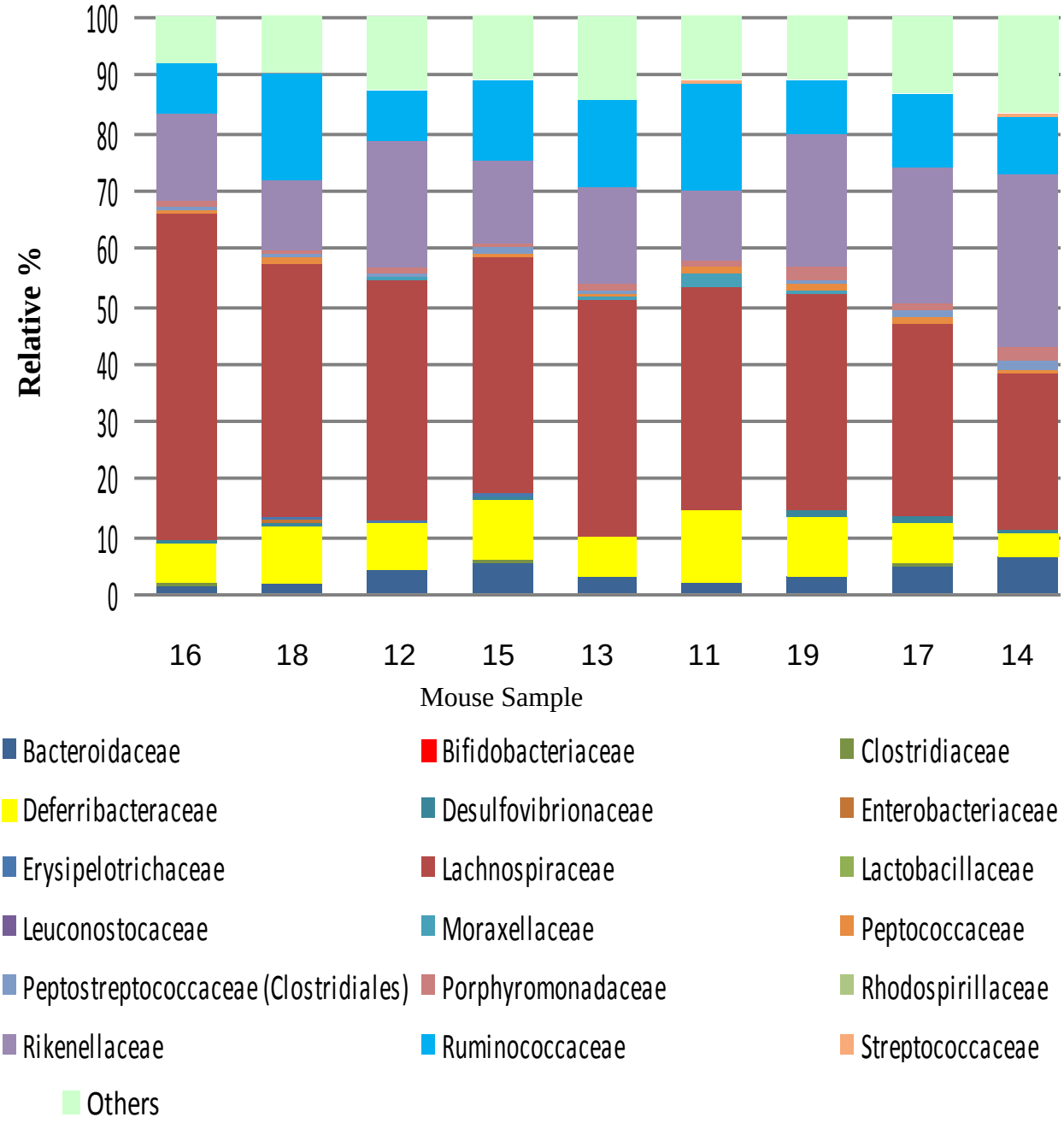

Figure 5.4 (d) Inter-individual variation in the proportion of the major family-level caecal microbiota of the mouse group fed α-linolenic acid enriched diet only. Samples are ordered from left according to *Firmicutes* proportion.

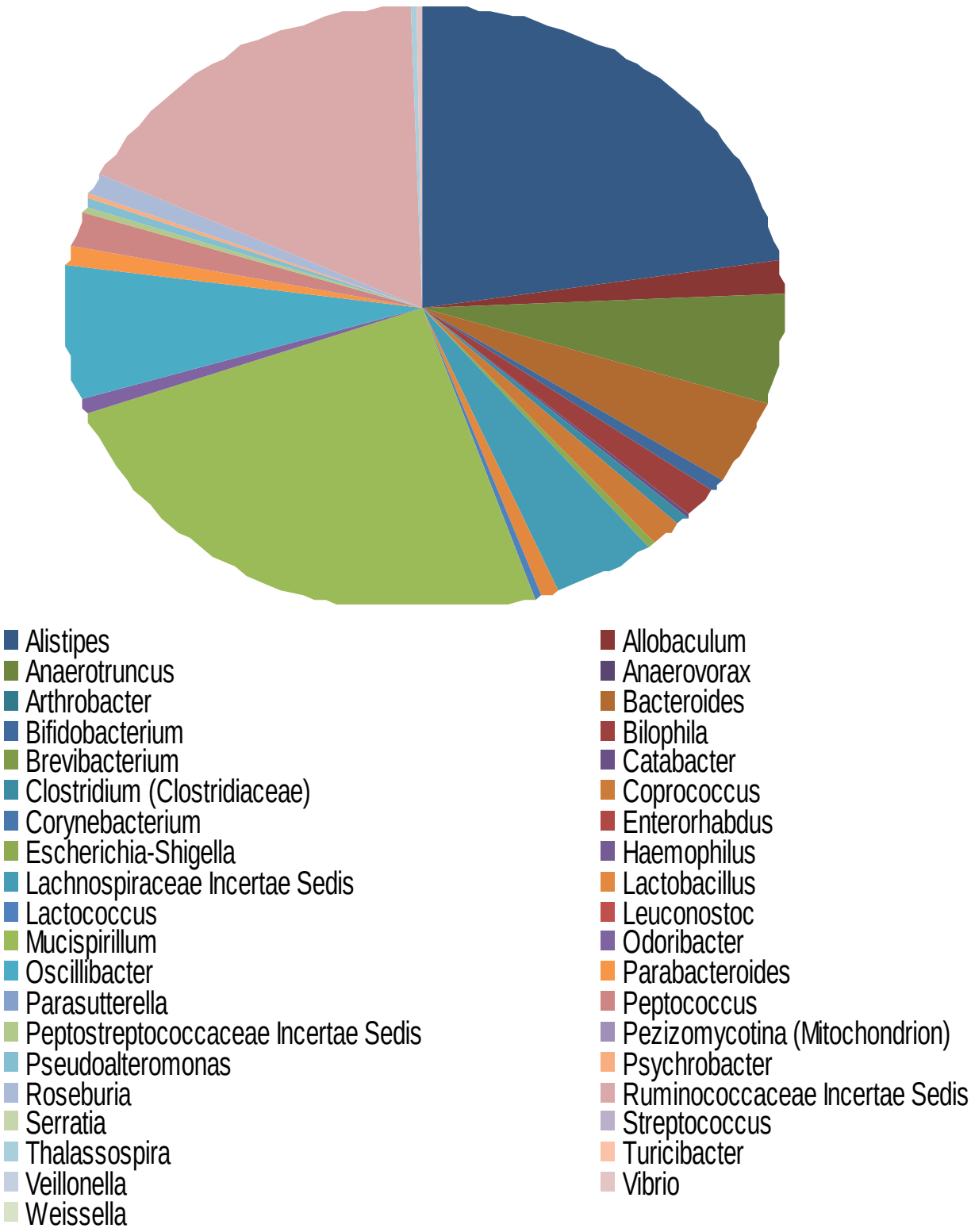

Figure 5.5 (a) The aggregate relative proportion of genera of the caecal microbiota of the mouse group fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315.

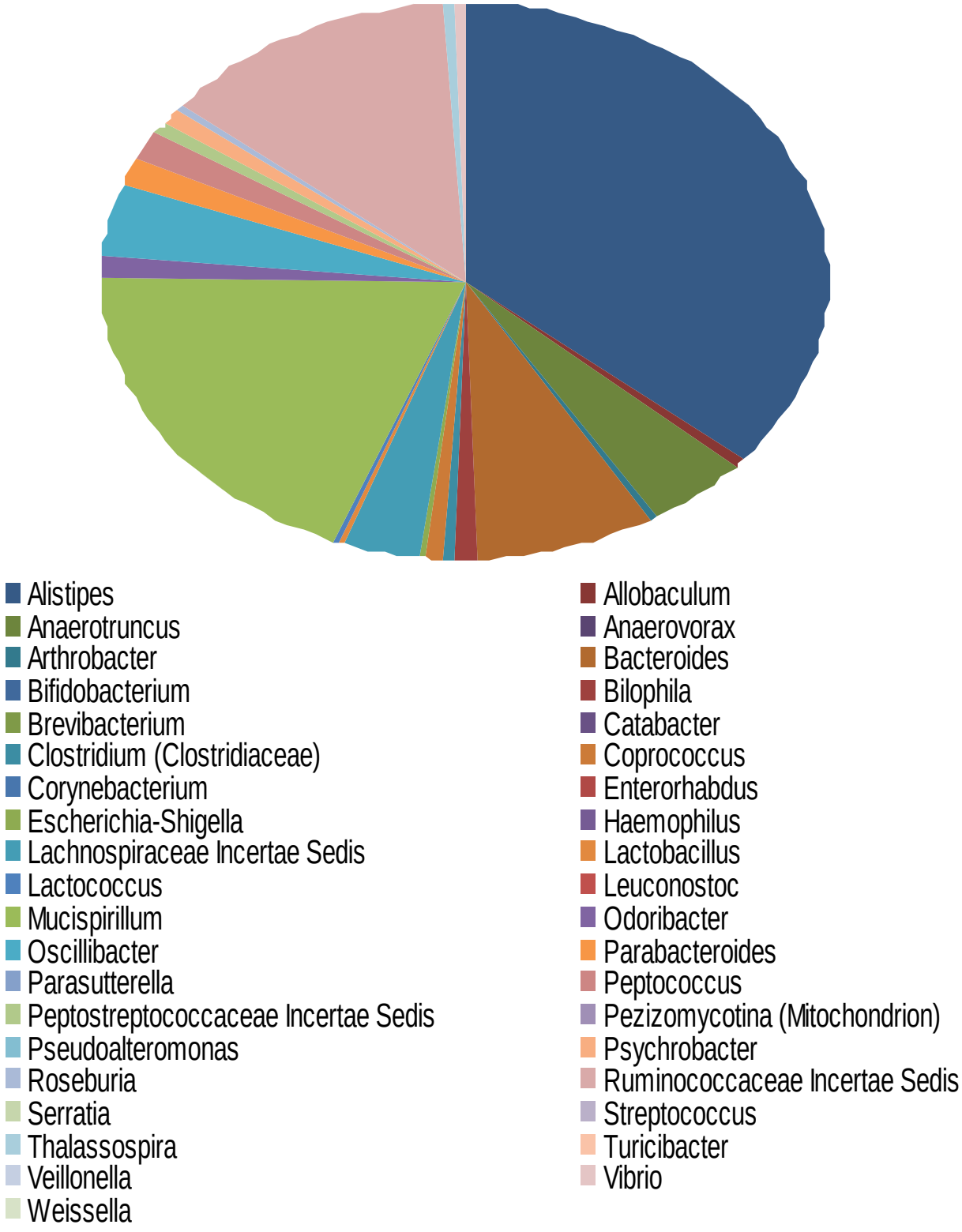

Figure 5.5 (b) The aggregate relative proportion of genera of the caecal microbiota of the mouse group fed α-linolenic acid enriched diet only.

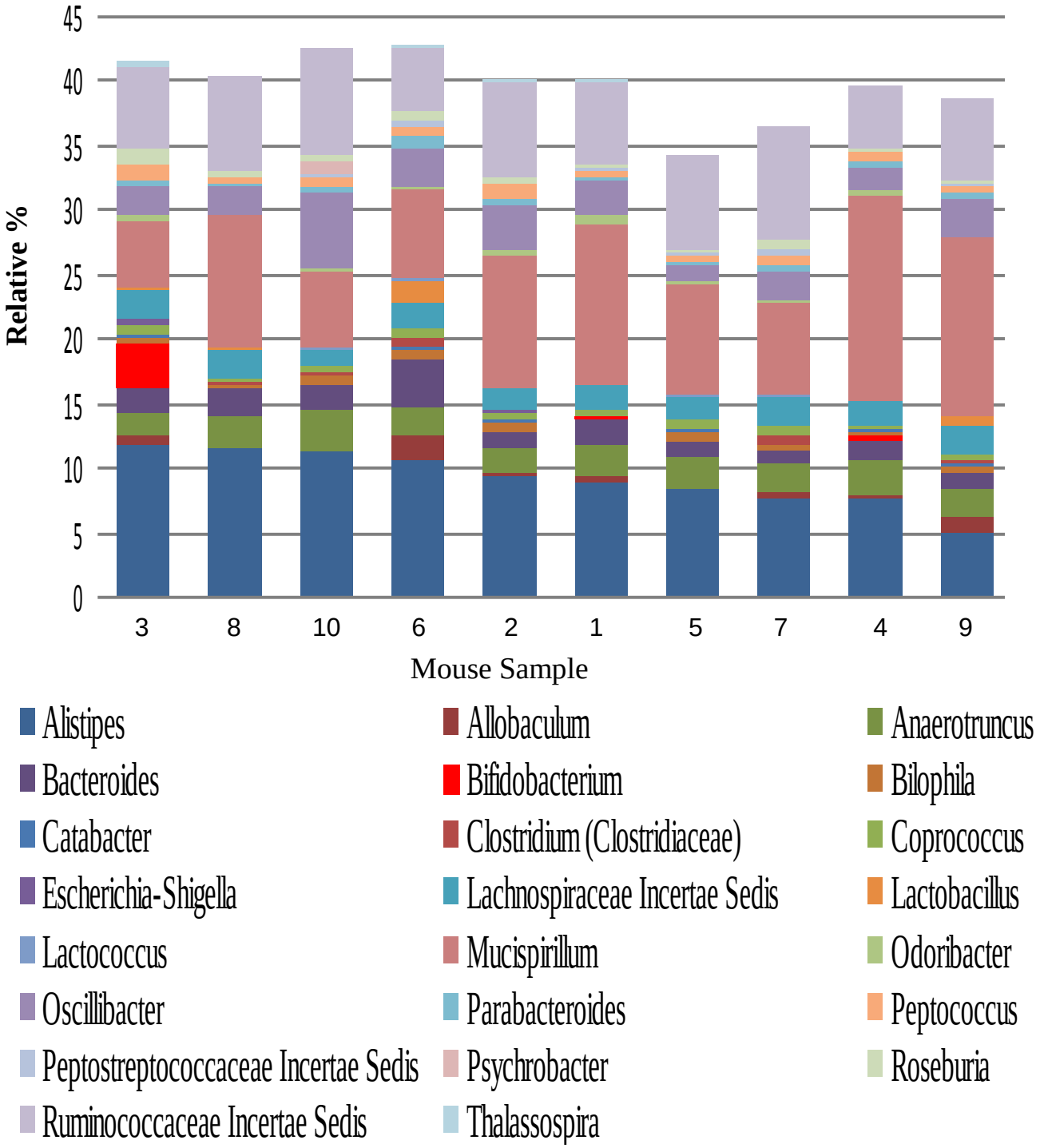

Figure 5.5 (c) Inter-individual variation in the proportion of genera of the caecal microbiota of the mouse group fed α-linolenic acid enriched diet supplemented with *B. longum* DPC 6315.

Samples are ordered from left according to *Firmicutes* proportion.

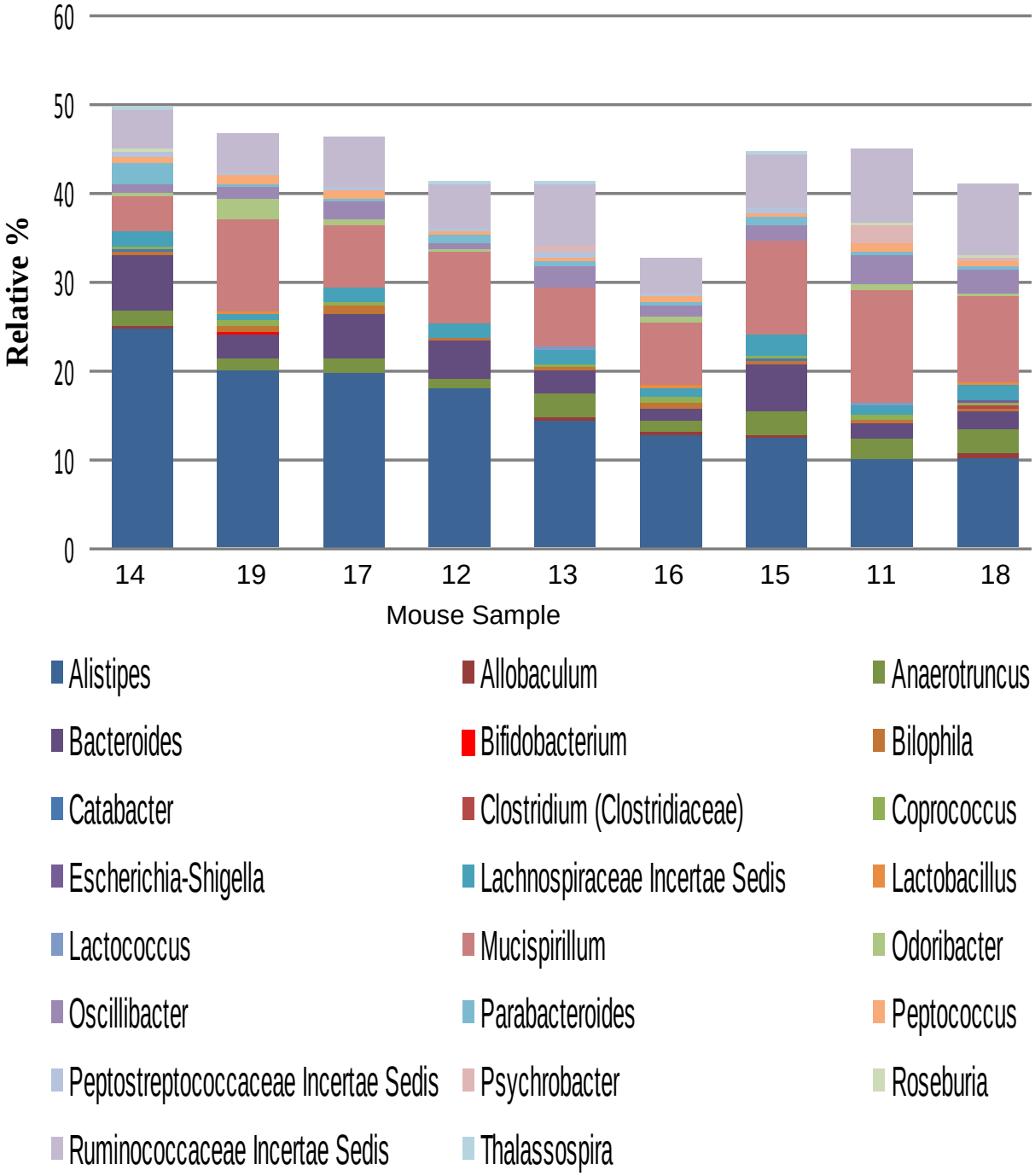

Figure 5.5 (d) Inter-individual variation in the proportion of genera of the caecal microbiota of the mouse group fed α-linolenic acid enriched diet only. Samples are ordered from left according to *Firmicutes* proportion.

CHAPTER 6

General Discussion

6.1 GENERAL DISCUSSION

Bifidobacteria are commonly used as probiotics for human consumption, and are normal inhabitants of the GIT of humans (Simon & Gorbach, 1984). They have a long history of safe use in fermented milks (Ishibashi & Yamazaki, 2001), and are commonly added to the human diet as probiotic supplements. Bifidobacteria have been reported to exert numerous positive effects on human health such as prevention of infection by pathogenic bacteria (Wang *et al.*, 2004) immunostimulation (Furrie *et al.*, 2005), anti-carcinogenic capabilities (Le Leu *et al.*, 2010), protection against infectious diarrhoea, (Saavedra *et al.*, 1994), lowering of serum cholesterol (Xiao *et al.*, 2003) and alleviation of lactose maldigestion (He *et al.*, 2008).

Bifidobacteria have been found to produce metabolites that have the capacity to exert a positive influence on host health. These metabolites include CLA (Coakley *et al.*, 2006), EPS (Salazar *et al.*, 2008) and bacteriocins (Lee *et al.*, 2011). The human intestinal strain *B. longum* DPC 6315 was examined in detail using both phenotypic and genomic approaches in this study. The strain produced both CLA and EPS and contained all the genes necessary for lantibiotic production. Other activities of bifidobacteria that can be viewed as having positive influences on host health are SCFA production (Lankaputhra & Shah, 1998) and the ability to utilize prebiotics that can help to increase beneficial gut microbiota (Cani *et al.*, 2007).

As bifidobacteria are naturally found in the human GIT, their presence and metabolic activities are generally considered integral to the maintenance of human health and subsequently well-being. They are found in the human GIT throughout life after appearing shortly after birth (van der Werf & Venema, 2001). There also appears to be a

general inverse relationship between bifidobacteria numbers and undesirable bacteria such as clostridia and *Escherichia coli* in the GIT as observed from clinical feeding studies (Alvaro *et al.*, 2007; Chen *et al.*, 1999), which would suggest that bifidobacterial metabolic activities have a role in preventing the growth in the GIT of these undesirable microorganisms. The utilization of non-digestible substrates by *Bifidobacterium,* such as complex carbohydrates including cellulose and starch provides the human host with a valuable energy source which would otherwise be lost from the body (Vaughan *et al*., 2005). Bifidobacteria, as an indigenous bacterium in many human hosts have developed a symbiotic relationship with the host as both the bacterium and the carrier benefit from the association. Bifidobacteria are provided with substrates and anaerobic conditions in the GIT to support growth, and the host benefits from the beneficial metabolic activities of the *Bifidobacterium* species that inhabit the GIT.

This thesis focused on the identification and characterization of certain potential probiotic traits of bifidobacteria isolated from the human GIT. One of these strains *B. longum* DPC 6315 was studied employing both genomic and phenotypic methods to examine in more detail its identified potential probiotic traits. In addition, the effect of *B. longum* DPC 6315 on the functional properties of yoghurt and on fatty acid metabolism and intestinal microbiota in a murine model was investigated. Great potential exists for the use of *Bifidobacterium* strains in future probiotic or functional foods, especially involving *Bifidobacterium* strains normally found in the GIT such as *Bifidobacterium longum*.

As many of the positive effects on human health associated with bifidobacteria are due to the production of metabolites such as CLA, EPS and lantibiotics, bifidobacteria of

human intestinal origin were screened, using a combined phenotypic and molecular approach in Chapter 2. 38 *B. longum* strains isolated from human infant and adult faecal samples were differentiated into subspecies using ARDRA analysis. Of the 38 *B. longum* strains tested, 34 were designated *B. longum* subsp. *longum* and four *B. longum* subsp. *infantis*. Phylogenetic analysis revealed that each of these 38 *B. longum* strains was distinct from one another and that the *B. longum* strains appeared to share 40% or higher genetic similarity. Certain DNA macro-restriction fragments were found in a high proportion of the 38 *B. longum* strains such as those at ~110 kb, ~120 kb, ~194kb, ~23.1 and at ~9.42 kb. These patterns would appear to be highly conserved regions of the genomes of the *B. longum* strains examined in this study.

The screening analysis revealed that 15 *B. longum* subsp. *longum* and one *B. longum* subsp. *infantis* strain produced an EPS phenotype and that 24 *B. longum* subsp. *longum* and two *B. longum* subsp. *infantis* strains had a corresponding genotype with the *wblE* gene (Audy *et al*., 2010). As such 12 strains appeared to have the genes but did not produce EPS – in these cases the genes may not be expressed, may be deleted or mutated. Ten of the 38 strains converted free linoleic acid to CLA and although lantibiotic production was not confirmed in the laboratory by the methods employed in this study, genetic analysis revealed that 6 strains were positive for the *lanA* gene. This is the first known study to report the production of both EPS and CLA in one bifidobacterial strain. In total, 3 *B. longum* strains produced both EPS and CLA, while the strain *B. longum* DPC 6315 produced EPS, CLA and whilst it did not produce lantibiotic *in vitro,* it contained the *lanA* gene necessary for lantibiotic production. For this reason, this strain was the main focus of Chapters 3, 4 and 5 of this thesis.

In Chapter 3, the genomic analysis of the human isolate *B. longum* DPC 6315 was performed. The genes for EPS, lantibiotic and genes believed to be involved in CLA production were identified in the draft genome of *B. longum* DPC 6315. The EPS produced by this bifidobacterial strain was found to be a unique EPS, with high levels of mannose which could possibly give the strain positive immunodulatory effects *in vivo*. The high levels of mannose in EPS produced by bifidobacteria have only been previously reported for three other *Bifidobacterium* species, i.e. *B. longum* DSM 21062, CHCC8773 and CHCC8818 (Kildsgaard *et al.*, 2011). This strain was reported to possess anti-inflammatory effects in a trinitrobenzene sulfonate (TNBS)-induced colitis model when fed to mice at 1×10^9 live bacteria daily for 5 consecutive days compared to the two other *B. longum* strains tested which were CHCC8773 and CHCC8818 (Kildsgaard *et al.*, 2011), causing a reduction in TNBS-induced injury by 44% (P=0.007). It has also been previously reported that complex polymers containing mannose (mannans) possess significant biological activity when administered to mammals, which includes the activation of the immune system following the binding of mannose to recognition molecules such as the mannose receptor (CD206) expressed on macrophages and dendritic cells (Carroll & Prodeus, 1998; Diebold *et al.*, 2002; Stahl & Ezekowitz, 1998). It is also possible that the EPS produced by *B. longum* DPC 6315 could have positive immunodulatory effects *in vivo*. An interesting future study would be to examine immunodulatory effects of the EPS produced by *B. longum* DPC 6315 in a murine model.

The lantibiotic gene operon identified in the genome of *B. longum* DPC 6315 was previously identified in *B. longum* DJO10A (Lee *et al.*, 2008). Using well diffusion assays and spot plate overlays, no activity of the *B. longum* DPC 6315 supernatants were

found against other *B. longum* strains (Chapter 2), or against various Gram-positive and Gram-negative pathogenic bacteria. This could be due to one of the genes essential for lantibiotic production not being expressed in *B. longum* DPC 6315 or that the level of the lantibiotic produced by the strain was too low to exhibit antimicrobial activity against indicator strains *in vitro*. It is possible that this lantibiotic would have an effect in the GIT *in vivo,* as there is more competition for nutrients and binding sites from other gut bacteria, and the production of a lantibiotic *in vivo* would confer an advantage on *B. longum* DPC 6315 over other bacterial strains present.

The focus of Chapter 4 was to examine the effect of exopolysaccharide producing *B. longum* DPC 6315 when used as an adjunct culture during yoghurt manufacture on the functional properties of yoghurt. The EPS producing bifidobacterial strain used to manufacture yoghurt in this study survived to the FAO/WHO minimum required levels of a probiotic culture (10^6 CFU/g) in the product after 28 days of cold storage, although it did have a 1000 fold reduction in viability - most likely due to the low pH of the yoghurt. *B. longum* DPC 6315 produced relatively low amounts of EPS in the yoghurt produced in this study, and the EPS produced did not significantly increase the viscosity of this yoghurt compared to the control - although both yoghurts displayed a shiny, slightly ropy appearance. Overall, this study demonstrated that the production of EPS by *B. longum* DPC 6315 in yoghurt reduced the syneresis of the EPS containing yoghurt up to day 14 of cold storage. The presence of this bifidobacterial strain in the yoghurt led to significantly higher levels of EPS compared to the control yoghurt, up to day 28 of cold storage. The levels of EPS significantly declined after day 7 of cold storage however, the strain was found to produce significantly higher concentrations of EPS when grown in

14% (w/v) RSM and 1% (w/v) yeast extract without the CH-1 yoghurt starter cultures. The wide range of EPS concentrations reported in yoghurt for different EPS producing strains could be due to the use of different strains, the level of inoculation, fermenting conditions, method of isolation, purification and quantification of EPS between studies (Amatayakul *et al*., 2006). With prolonged storage, a decrease in EPS-concentrations for the EPS-containing yoghurt was reported by Purwandari et al. (2007), where the EPS concentrations in all batches of yoghurt made with two different strains of *Streptococcus thermophilus* declined significantly ($P<0.05$), especially in the first week. The significant ($P<0.05$) reduction of the EPS concentration reported in these yoghurt batches during the first weeks of cold storage could be due to the activity of enzymes capable of degrading EPS (Degeest *et al*., 2002).

It is possible that EPS degradation may be activated due to the cell's requirements for energy maintenance, as the EPS may serve as an energy store in the presence of excess nutrients, which may be used later in the cell metabolism (Tolstoguzov, 2003). Many different studies have demonstrated the reduction in EPS concentrations after prolonged incubation of *S. thermophilus* (Cerning, 1990; Cerning *et al.*, 1988; De, 1998; De Vuyst, 1999; Gancel & Novel, 1994; Macura & Townsley, 1984; Pham *et al.*, 2000). It has been suggested that EPS degradation during fermentation is due to a change in the physical or chemical factors linked to glycohydrolase activity. In the study performed by Pham et al. (2000), a greater reduction in EPS concentration was found in the case of lactose-grown *Lactobacillus rhamnosus* R cells than in glucose-grown cells. The EPS producing culture had a positive effect in reducing syneresis in the yoghurt on day 0, 1, 7 and 14 of cold storage with no significant difference in syneresis between the control and

EPS containing yoghurt on days 21 and 28. This is possibly due to the degradation of EPS in the EPS containing yoghurt with a reduction of EPS on day 14, 21 and 28 of cold storage, respectively. Syneresis is considered a major defect in the yoghurt industry and is directly related to the extent of physical disturbance to the network of protein micelles (Tamime & Robinson, 2007). It has previously been reported that ropy EPS has a greater ability to retain serum, resulting in lower syneresis in yoghurt (Folkenberg *et al*., 2005; Lucey *et al*., 1998). Some other studies have previously reported that the intensity of syneresis decreased in set-type yoghurts during prolonged storage (Guzel-Seydim *et al*., 2005; Kearney *et al*., 2009; Purwandari *et al*., 2007; Robitaille *et al*., 2009). The EPS containing yoghurt in this study exhibited a significant reduction in syneresis up to day 14 of storage compared to the control which indicates that at a certain concentration the EPS produced by *B. longum* DPC 6315 has the ability to bind free water and reduce syneresis in yoghurt.

The EPS produced by *B. longum* DPC 6315 after 28 days of cold storage was well distributed and mainly located around the edges of serum pores of the yoghurt, connected with the protein network as revealed by CLSM microscopy. A similar microstructure was also reported in the studies performed by Folkenberg et al. (2005) and Kearney et al. (2009), where an overlap of protein and EPS was observed and the yoghurt product was described as being very shiny, very ropy, with low serum separation and was resistant to mechanical treatment, which correlates positively with the results of this study. The extent of the interaction between the EPS produced by *B. longum* DPC 6315 and the yoghurt network is unknown, however the association between EPS and protein appears to be positive, as incompatibility between EPS and protein would lead to phase

separation, with EPS being expelled into the serum phase (Hassan *et al.*, 2003). In future studies, it would be worthwhile to examine the effects of encapsulated *B. longum* DPC 6315 cells in a fermented food. The encapsulation of the bacterial cells would ensure a higher survival rate, which may also lead to higher levels of EPS being produced with improved functional effects on the texture and syneresis of the food product.

In Chapter 5, the effects of feeding *B. longum* DPC 6315 in a high fat diet enriched with α-linolenic acid on intestinal microbiota and fatty acid metabolism in a murine model was investigated. Dietary supplementation with *B. longum* DPC 6315 was found to have significant effects on fatty acid composition of the murine liver and brain. The group fed a high fat diet enriched with α-linolenic acid together with *B. longum* DPC 6315 was found to have significantly lower levels of linoleic acid and γ-linolenic acid in the liver, and stearic acid levels in the brain compared with unsupplemented mice. In addition, mice that received *B. longum* DPC 6315 had significantly higher concentrations of stearic acid, EPA and DPA in the liver, compared to unsupplementated animals. The study previously performed by Wall et al. (2010), also reported similar findings, where *B. breve* NCIMB 702258 and the substrate for EPA and DHA, α-linolenic acid was fed to mice, with the control group receiving α-linolenic acid only. Significantly ($P<0.05$) higher levels of EPA in the liver and DHA in the brain were found following bifidobacteria supplementation compared with the control group. Eicosanoids from EPA are reported to be less inflammatory than those from arachidonic acid or even anti-inflammatory in nature (Bagga *et al.*, 2003; Calder, 2008; Robinson & Stone, 2006). EPA can be further metabolized to DHA, which has a vital role in fetal brain development and is critical in the newborn for optimum development and cognition (Salem *et al.*, 1996). It

has been demonstrated in some studies that dietary DHA contributes to improved learning and memory events in animal models of Alzheimer's disease (Hashimoto *et al.*, 2002) and brain injury (Wu *et al.*, 2004). It is possible that the presence of *B. longum* DPC 6315 is causing the increases in both EPA and DHA in the liver and that this bacterial strain has the potential to positively influence the overall fatty acid composition of the host.

In the mouse group supplemented with *B. longum* DPC 6315, *Firmicutes* were found at a significantly higher relative abundance in the caecum, while *Bacteriodetes* were found at a significantly higher proportion in the unsupplemented group. Some studies have found *Firmicutes* at significantly higher relative abundances in obese compared to lean subjects (Ley *et al.*, 2005; Ley *et al.*, 2006; Turnbaugh *et al.*, 2006). There is no reason to suggest that this is the case with the feeding of *B. longum* DPC 6315, as there were no observed differences in animal weight or fat composition between the groups. There were also significant differences at the genus level as *Alistipes* was detected at a significantly lower relative abundance in the *B. longum* DPC 6315 supplemented group. The *Alistipes* genus is closely related to the genus *Bacteroides* and contains species such as *Alistipes finegoldii* which has been found to cause bacteremia in colon cancer patients who had undergone surgical resection (Fenner *et al.*, 2007) - so a significantly lower relative abundance of this genus could be interpreted as a positive outcome, although future studies are warranted to confirm this. *Roseburia* was detected at a significantly higher relative abundance in the supplemented group and this genus has been reported to contain species that produce butyrate as a fermentation end-product, which has been shown to be a major source of energy for colonic epithelial cells

(Cummings, 1981; Cummings *et al*., 1987; Roediger *et al*., 1982). Butyrate provides the body with 7-10% of its energy needs (Roy *et al*., 2006), and has also been shown to have anti-carcinogenic effects by preventing mutagens at their active site, molecular or DNA level (Smith, 1995). It is possible that the supplementation of *B. longum* DPC 6315 is leading to a higher relative abundance of this genus which contains butyrate producers. Chapter 5 demonstrated that supplementation with *B. longum* DPC 6315 led to significant differences in fatty acid composition in murine liver and brain compared with supplementation with the high fat diet only. *B. longum* DPC 6315 supplementation also led to significant differences in the overall caecal microbiota at both the phylum and genus levels.

Overall, the data in this thesis demonstrates that certain bifidobacterial strains can produce EPS and CLA. *B. longum* DPC 6315 also possesses the potential for bacteriocin production. The strain can also produce significant levels of EPS and significantly improve some of the techno-functional characteristics of yoghurt. It was also shown that *B. longum* DPC 6315 can modify the fatty acid composition in a murine model, producing significantly higher levels of the anti-inflammatory fatty acids, EPA and DHA in the liver. *B. longum* DPC 6315 also influenced the murine caecal microbiota, with a significantly higher relative abundance of *Firmicutes* and a significantly lower relative abundance of *Bacteriodetes* in the caecal microbiota of mice fed this strain compared to mice that did not receive *B. longum* DPC 6315. Supplementation with *B. longum* DPC 6315 also led to a higher relative abundance of the genus *Roseburia* (containing some beneficial species) and a significantly lower relative abundance of *Alistipes* (containing some harmful species) in mice that received the bacterium compared to unsupplemented

mice. These various positive attributes of the strain make it a very attractive candidate for future probiotic use in humans. The next step in this progression would involve human intervention trials with a probiotic yoghurt where ingestion of the strain would be examined for its effect on the gut microbiota and human fatty acid metabolism and ultimately on human health.

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